5-2016

Computational Perspective for Developing Bioluminescent Yeast Estrogen Screens for Environmental Toxicology

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To the Graduate Council:

I am submitting herewith a dissertation written by Jun Wang entitled "Computational Perspective for Developing Bioluminescent Yeast Estrogen Screens for Environmental Toxicology." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Gary S. Sayler, Major Professor

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Computational Perspective for Developing Bioluminescent Yeast Estrogen Screens for Environmental Toxicology

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Jun Wang

May 2016

Dedication

To all the people that love me and I love.

Acknowledgements

The journey of my PhD study won't be possible without the help from many people. First of all, I want to thank my mentor Dr. Gary Sayler for his generous support, encouragement and guidance during my study at Center for Environmental Biotechnology. I am very grateful for his patience and encouragement when I encountered difficulties and challenges. His dedication, vision and passion on science, as well as his genuine personality will always be my inspiration of what a great scientist should be. I also thank my doctoral committee members, Dr. Alison Buchan, Dr. Erik Zinser, Dr. Jerome Baudry and Dr. Qiang He for their suggestions and constructive feedbacks. I would like to give special thanks to all the colleagues and friends that I met and worked with at University of Tennessee, specifically Melanie Eldridge, Fu-min Menn, Jie Zhuang, Thomas Mead, Tingting Xu, Alice Layton, Steven Ripp, James Fleming. Lastly, I thank my parents and my wife Xiaoxin for their unconditional love and support during these years.

Abstract

The impact of endocrine disruptive chemicals to human and wild life health has raised serious public health concerns through the past decades. To address this concern, much research was involved to develop tools for screening and assessing the hormonal potential of these compounds. Yeast bioluminescent bioreporter assay was one of the tools developed as the result of these past research endeavors. In this dissertation, a yeast bioluminescent bioreporter assay system was evaluated for the screening of endocrine disruptors from both experimental and computational perspectives. The yeast bioluminescent bioreporters were first standardized and applied in the comparative study of traditional activated sludge and membrane bioreactor wastewater treatment facilities for their performance in endocrine disruptor removal. Then the interaction between endocrine disruptors and their target, human estrogen receptor (hER), was studied by both computational modelling and experimental approaches. Specifically, the effects of naturally occurring mutations on hER were investigated for their interaction with 29 estrogenic endocrine disruptors through molecular dynamics simulation and virtual docking. To verify the predicted results from computational modelling, new yeast bioluminescent reporters harboring the mutated hER were constructed to evaluate the hER-mediated transactivation triggered by 12 selected endocrine disruptors. hER mutations caused various degree and pattern of changes in the response to the 12 tested endocrine disruptors in the yeast bioassay. The potential mechanism for the altered ER mutant response and their possible health related impacts were discussed.

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Chapter 1. Introduction and Literature Review

Introduction

Human beings are exposed to a broad spectrum of chemicals that have hormonal potencies. Since these hormonally active compounds can directly impact and interfere the normal function of the endocrine system, they are commonly referred as endocrine disruptive chemicals (EDCs). In recent decades, substantial evidence has been developed that associates the exposure of EDCs to various human diseases ranging from breast cancer to osteoporosis to developmental disorders(Diamanti-Kandarakis, Bourguignon et al. 2009). Therefore EDCs have raised significant public health concern and become a challenging problem to both the government regulators and scientific community. Due to the prevalence of EDCs in the environment, there is an urgent need to understand the fate, transport and transformation of EDCs in the environment, and how they can affect human and animal health. With these central questions, this dissertation provides a critical development of the application of yeast EDC bioluminescent reporters in the study of environmental EDC biotransformation in wastewater treatment, and a computational and experimental perspective on human estrogen receptor mutants and their interaction with EDCs.

The central hypotheses tested in this dissertation are: 1). the hormonal potential of EDCs are modulated when undergoing the biotransformation process in wastewater treatment and 2). natural genetic variation in human estrogen receptor can lead to altered susceptibility and response to different EDCs.

Chapter 2 focuses on the first hypothesis, applying the yeast bioluminescent assay as a high-throughput screening tools to monitor the estrogenic, androgenic potencies and toxicity in the wastewater treatment systems. The study firstly evaluated the sensitivity and reproducibility of

the yeast assay through a 7 day semi-continuous batch reactor experiments using the activated sludge and raw wastewater spiked with hormones. Then this standardized assay was applied in a 12 month comparative study on the wastewater effluent from the parallel-operated membrane bioreactor (MBR) and traditional activated sludge (TAS) located at Powell, TN, USA.

Chapter 3 and 4 focus on the second central hypothesis, and specifically examine five selected human estrogen receptor mutants and their interaction with EDCs from both computational and experimental perspective. As human estrogen receptor alpha (hERα) features a flexible ligand binding pocket that may bind many EDCs and modulate the activation of the receptor, research efforts were concentrated on evaluating the response of the most common receptor form, the "wild type" human estrogen receptor, that is defined by current understanding of the hERα protein. However in human genome, small genetic variations, often referred as single nucleotide polymorphism (SNPs) are found to be ubiquitously present. Previous studies have revealed numerous hERα mutations on the gene ESR1 in both healthy and cancer patients with primary and metastatic tumors(Roodi, Bailey et al. 1995, Segal and Dowsett 2014, Thomas and Gustafsson 2015). As these mutations found in ER originated from very limited population, and the incidence of mutations are far from uncommon, the past EDCs evaluation studies focusing solely on "wildtype" ER can be incomplete and biased. Consequently this raises a serious issue as to whether a susceptible subpopulation of hER mutants exist that demonstrates altered susceptibility or responses to estrogenic and xenoestrogenic chemicals in the environment. Therefore the studies in Chapter 3 and 4 specifically investigate five mutations of hERα inside or close to the ligand binding pocket for their effect to the interaction between a broad spectrum of estrogenic EDCs and the protein receptors. The key questions that were asked are: 1). Does a

mutation occurred inside or outside the ligand binding pocket of hER can cause differential response to estrogenic EDCs? 2). If a mutation on hER causes change of response to natural estrogen E2, will the direction and degree of deviation also apply to other estrogenic EDCs? To address these two questions, both computational modelling and experimental yeast bioluminescent estrogen assay were used to evaluate the binding and transactivation activity of wildtype and mutant hERα in the presence of EDCs from a wide range of origins (Fig 1.1). It is hypothesized that binding affinity changes predicted by computational molecular dynamics (MD) and ensemble docking simulations can provide guideline for experimental transcriptional activity evaluation using the yeast bioluminescent estrogen reporter.

In Chapter 3, two forms of hERα ligand binding domain, the agonist form and antagonist form, and five *in silico* mutated models were subjected in molecular dynamics (MD) simulation. MD trajectories were clustered to select snapshots from each of the cluster, which were then computationally docked with a list of 29 estrogenic ligands to evaluate the binding affinity changes as the result of the mutations.

In Chapter 4, several yeast bioluminescent estrogen reporters using different source of wildtype hER gene and bioluminescent reporter plasmids were newly constructed. The performance of these bioreporters was compared and a stable construct was selected and further optimized for its EDCs testing protocol. Based on this bioreporter construct, five bioreporters containing separately the five hERα mutations were constructed. A total of 12 EDCs including the natural estrogen 17β-estradiol (E2), synthetic hormones, plasticizer, phytoestrogen and industrial compound were evaluated with the wildtype and mutant hER yeast bioreporters.

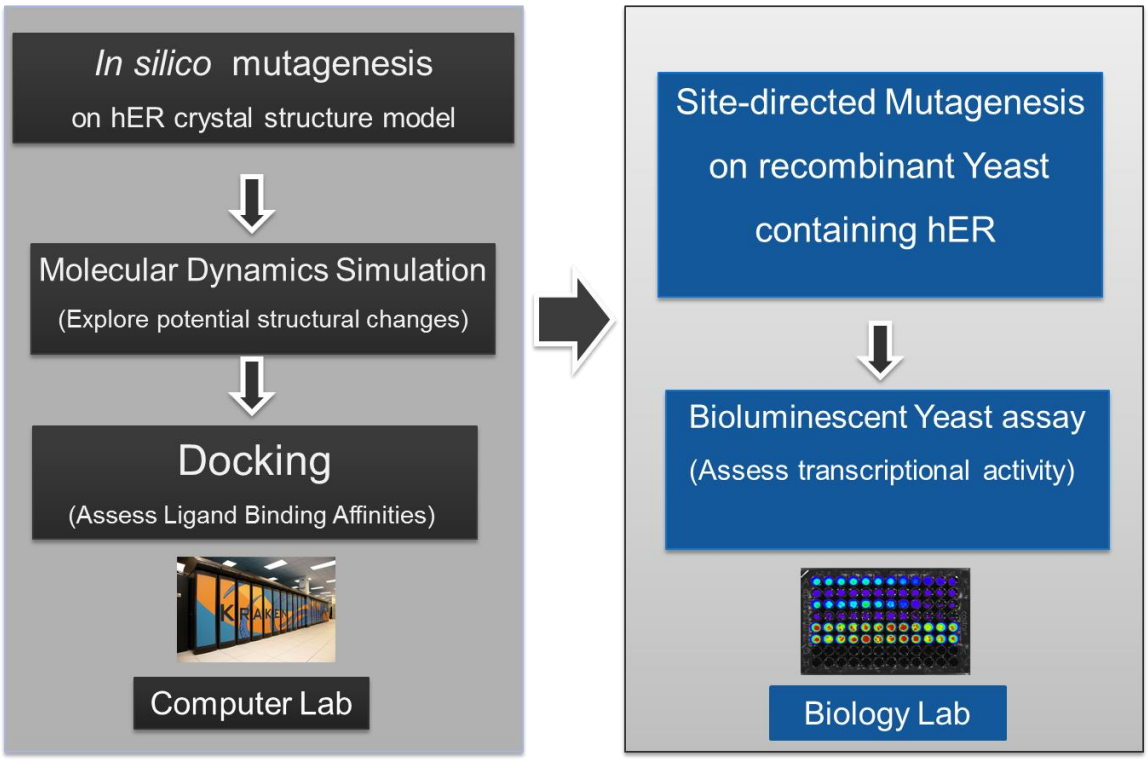

Fig 1.1. Scheme of the combined working flow for estrogen receptor mutant and EDCs interaction study using computational modelling and experimental approaches.

Literature Review

1.1 The Problem: EDCs in the environment

Magnitude of the problem

Endocrine disruptive chemicals (EDCs) refer to natural and synthetic compounds that can cause a variety of adverse health effects in human and animals by interfering with the normal function of endocrine system. EDCs are highly heterologous, consisting of a broad spectrum of compounds with a wide range of origins and diverse structures.

Commonly known EDCs included natural hormones such as phytoestrogen genistein, coumestrol, resveratrol, synthetic pharmaceutical such as 17α-ethinyl estradiol (EE2), diethylstilbestrol (DES), tamoxifen, plasticizers such as bisphenol A (BPA), pesticides such as dichlorodiphenyltrichloroethane (DDT), and industrial materials such as nonylphenol etc.(Diamanti-Kandarakis, Bourguignon et al. 2009). Due to the large quantity of synthetic chemicals being produced by today's industry, and the fact that a compound's endocrine disruptive potential cannot be readily determined by its structural property, the demand for potential EDCs identification and monitoring is enormous. To address the screening request enforced by The Federal Food, Drug, and Cosmetic Act (FFDCA 1996), and the Safe Drinking Water Act Amendments (SDWA 1996), United States Environmental Protection Agency (EPA) initiated the Endocrine Disruptor Screening Program in 1998 to investigate manufactured chemicals for endocrine disruptive potential. EPA estimated that over 87,000 new and existing chemicals require screening for potential endocrine disruptive activities(ICCVAM 2002), a scale that poses significant challenge to both the scientific community and the government regulators.

As EDCs come from very diverse sources, their presence in the environment are nearly ubiquitous. Since the scientific community reached a general consensus about the potential health impact of EDCs in early 1990s, many following investigations have been conducted to trace the presence of EDCs in the environment and the potential exposure pathway to human and animals(Colborn and Clement 1992). For human beings, exposure to EDCs are primarily through oral consumption, skin contact or inhalation. Through oral consumption, humans are exposed to EDCs such as residual pesticides on food (e.g. vinclozolin), leached chemicals of packaging (e.g. bisphenol A), phytoestrogens in soy-based food and dietary supplements (e.g. genistein, resveratrol), synthetic hormones in contraceptives and hormone therapy drugs (e.g. EE2, tamoxifen) etc. Skin contact is another route of exposure to the EDCs present in cosmetics, personal care products etc (e.g. phthalates, triclosan). Certain EDCs such as flame retardants brominated diphenylethers (BDEs) can also enter human body through inhalation (Diamanti-Kandarakis, Bourguignon et al. 2009). Compared to human beings, wild animals have high chance of exposure to EDCs released into the environment due to human activity. Numerous previous studies have reported that many natural and synthetic EDCs are persistent at various level in soil, sediment, surface and ground water, and even drinking water (Kolpin, Furlong et al. 2002, Lintelmann, Katayama et al. 2003, Ying and Kookana 2005, Benotti, Trenholm et al. 2008, Focazio, Kolpin et al. 2008). The contamination of EDCs in the aquatic environment has the most impact on aquatic organisms due to persistent exposure to EDCs in the habitat, while other animals exposed by the contaminated water or prey are also affected. Investigating and controlling the contamination of environmental EDCs is a rather demanding task since EDCs in the environment consists of a mixture of natural and synthetic hormones, and industrial compounds, and their occurrence, fate, transition also varies

depends on the specific geological location. The release of environmental EDCs can be mainly attributed to municipal and industrial wastewater effluent, agricultural runoff, occasional chemical spills and commercial product use and disposal. Among these sources, wastewater effluent contributes to the majority of environmental EDCs, as incomplete removal of organic contents in industrial and municipal sewage lead to continuous discharge of hormonally active compounds into the downstream watershed(Liu, Kanjo et al. 2009).

Health Impact of EDCs to human and wildlife

The human endocrine system includes a series of glands spread across the entire body (Fig. 1.2). These glands are responsible for producing a variety of hormones, which are small molecules circulated throughout the body to regulate critical physiological functions. The way hormones exert regulatory function is by binding to its specific protein receptors in the targeted tissues, triggering a variety of responses. Through the endocrine system, many critical functions ranging from homeostasis, metabolism, sexual development, reproduction, gender behavior etc. are maintained (Diamanti-Kandarakis, Bourguignon et al. 2009). EDCs could affect the endocrine system in several ways that either mimic natural hormones to bind to the receptors, block the receptors, or interfere the production/depletion of natural hormones/receptors. The endocrine system consists of complex components and mechanistically each type of hormones produced in the endocrine system plays specific and unique roles in maintaining the normal function of human body. Thus potentially any physiological function that relies on the regulation of endocrine system can be affected by EDCs.

The discovery of EDCs' effect on human health can be traced back to the usage of the synthetic hormone diethylstilbestrol (DES) since 1940, which was initially prescribed for preventing miscarriage in women. DES was banned by Food and Drug Administration (FDA) in 1971 due to the increased developmental birth defects and incidences of vaginal cancer in many women that received the treatment(Marty, Carney et al. 2011). As man-made chemicals being widely used in the industry and agriculture, a growing number of studies reported abnormal development and behaviors in wild animals (bird, fish etc.) including decreased fertility, decreased hatching success, feminization of male and defeminization of female etc., which was later attributed to the exposure of environmental EDCs in their habitats(Colborn and Clement 1992, Colborn, vom Saal et al. 1993). Since then, EDCs have been associated with numerous hormone mediated problems such as reduced reproducibility, birth defects, abnormal development, osteoporosis, obesity, breast cancer, etc.(Soto and Sonnenschein 2010, De Coster and van Larebeke 2012, Frye, Bo et al. 2012, Spencer, Dunlap et al. 2012). EDCs' adverse effect is of particular concern for fetus and newborn, as the hormonal balance in this critical stage of life is very delicate and interference from EDCs could possibly cause permanent effect(Haddow, Palomaki et al. 1999).

Microbial reduction of EDC potential in wastewater treatment

Wastewater effluent is the largest source of EDCs discharged into the environment. Since the EDCs contained in the wastewater effluent can lead to further contamination into the surface water, ground water, sediments and soil etc., effective removal of EDCs from wastewater at the treatment facilities is pivotal. Generally EDC removal at wastewater treatment plants (WWTP) can be classified into three categories : 1) physical separation such as activated carbon adsorption, membrane rejection, 2) biotransformation and degradation by concentrated microbial biomass,

3) chemical advanced oxidation by O_3, H_2O_2 etc.(Liu, Kanjo et al. 2009). However, due to the high cost of physical separation and chemical oxidation technology, most WWTPs rely on concentrated microbial biomass, activated sludge, to metabolize and remove organic pollutants including EDCs. Historically, the commonly used activated sludge in WWTP was primarily designed with aim of removing phosphorus and nitrogen contents in the wastewater. Therefore as EDCs discharge become a serious environmental concern, many studies reexamined the processing of WWTPs regarding their performance on trace organic chemical and EDCs removal. The reported data from both field WWTP monitoring and lab-scale simulation experiments suggest that EDC removal by current WWTP processing is incomplete, and the efficiency of EDC removal varies greatly depending on the type and level of influent EDCs, technical setting and operational parameters of the WWTPs(Liu, Kanjo et al. 2009). Some chemicals such as estrone (E1) and 17β-estradiol (E2) can have over 90% removal, while other chemicals such as 17α-ethynylestradiol (EE2), nonylphenol etc. are more persistent in the wastewater treatment system(Clara, Strenn et al. 2005, Drewes, Hemming et al. 2005, Chimchirian, Suri et al. 2007). As EDCs persist in the effluent of WWTPs, much research has focused on improving the efficiency of EDC removal in wastewater treatment and improving monitoring protocols. Apart from optimizing WWTP operational parameters such as mixed liquor suspended solid (MLSS) concentration, solid retention time (SRT), hydraulic retention time (HRT) etc, new secondary or tertiary treatment technologies such as membrane bioreactors, ozonation and advanced oxidation processes, activated carbon adsorption etc. were developed and studied for their EDC removal efficiency(Chang, Choo et al. 2009, Luo, Guo et al. 2014). Since EDC removal efficiency can be affected by the treatment technologies as well as the properties and contents of the

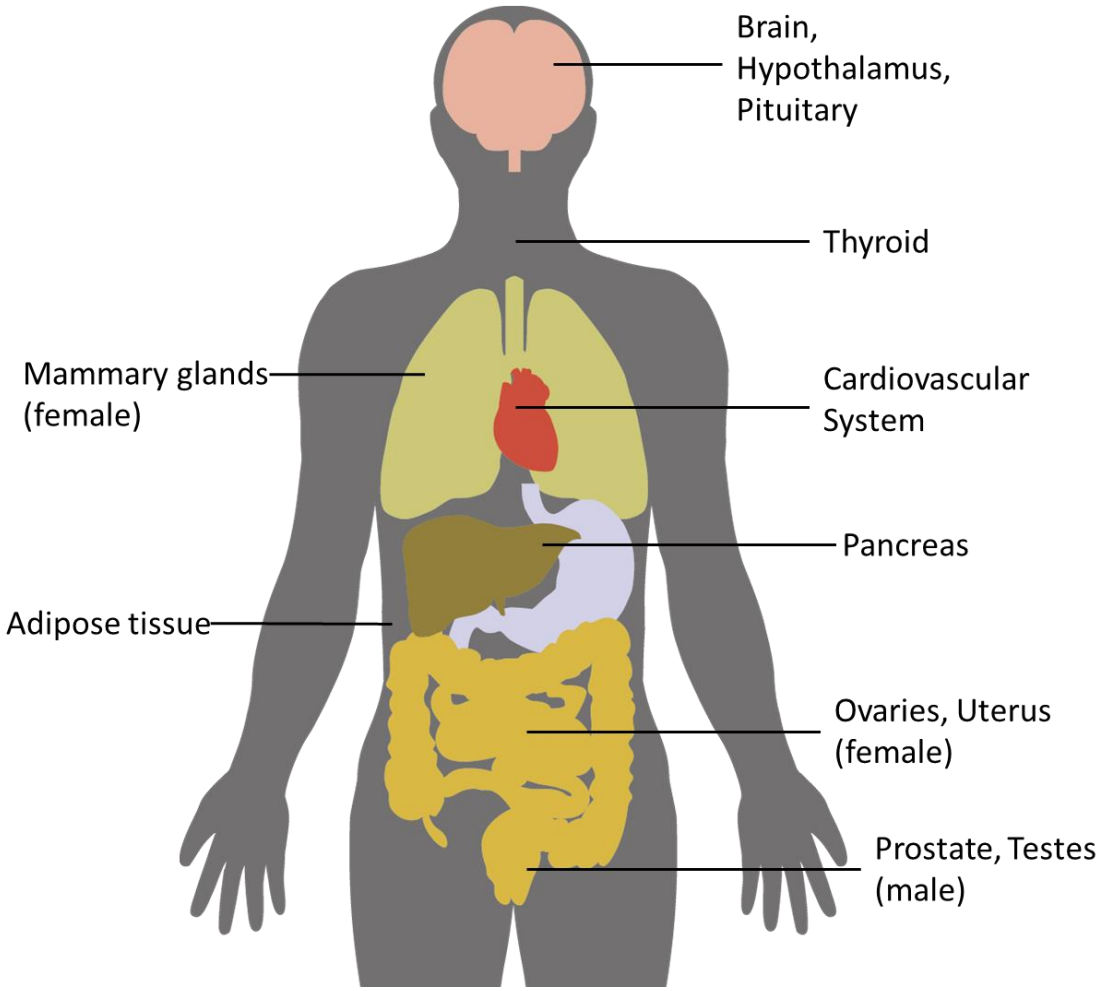

Fig 1.2. Diagram of major endocrine glands in human body. Adapted from (Diamanti-Kandarakis, Bourguignon et al. 2009).

wastewater, effective screening tools are required to monitor and evaluate the performance of WWTPs. During wastewater treatment, reduction of a parent compound doesn't necessarily result in reduced biological activity as the biotransformed products could still have potent or even augmented hormonal activity. Therefore when assessing the EDC removal efficacy in wastewater treatment, besides tracking the fate of the targeted compound, it is necessary to evaluate the changes in biological activities as well.

Screening assays and analytical methods for EDCs

Typically, detection of EDCs can be achieved by traditional mass-based methods (i.e. gas chromatography or liquid chromatography coupled mass spectrometry etc.), or biologically based methods. Biological assays were developed in a variety of forms that can be classified into three general categories, *in vivo* whole organism assays, *in vitro* cell-based assays and *in vitro* cell-free assays.

To determine the endocrine disrupting potential of a compound, the ideal methodology is through *in vivo* toxicological exposure experiments with whole organism models. However, the large quantity of chemicals that require screening for endocrine disruptive potential make it impossible to rely on animal models due to time, fiscal constraints and animal welfare concerns. To tackle this challenge, U.S. EPA proposed a two-tiered screening program, with tier 1 focuses on high-throughput priorization of chemicals that have potential to interact with the endocrine system, and tier 2 further determines the specific effects and dose response caused by each identified chemical(Rotroff, Dix et al. 2013). In response, the scientific community developed a variety of *in vitro* and *in vivo* assays to fulfill the need of tier 1 and tier 2 screenings. These assays

are not only used for single chemical testing, but are also widely used for evaluating the biological activity of mixture EDCs and environmental sample monitoring.

Traditional analytical methods usually employ mass spectrometry to provide targeted analysis of compounds available in the database. Typically, mass-based method requires sample extraction/concentration through liquid/solid phase extraction. In sample detection, mass spectrometry is usually coupled with sample separation instrument such as liquid chromatograph or gas chromatograph(Chang, Choo et al. 2009). As a widely used analytical tool, mass-based methods have high sensitivity, high specificity and excellent quantitative ability, therefore very suitable for detecting trace level EDCs in samples. Nevertheless, mass-based detection requires costly investment on specialized equipment and personnel training, hindering its application for large scale screening tasks. In addition, mass-based methods rely on pre-knowledge of a chemical's structural property and is unable estimate the biological activity of EDCs, making it less practical especially when the sample contains unknown or a mixture of EDCs. In these cases, screening with biological assays are necessary to ensure comprehensive evaluation of the endocrine disruptive activities.

Currently studies on the biological effects of EDCs primarily focus on their interference with the estrogen, androgen and thyroid hormone mediated functions, though estrogen related studies account for the majority because the number of known estrogen related health problems seem to far exceed the others. To study the hormonal activity of a specific compound, both *in vitro* and *in vivo* assays are commonly used, with each offering distinct advantages. *In vivo* assays employ model animals such as amphibian, fish or mouse to evaluate EDCs' biological effect. This type of assay monitors the organism's changes in morphology such as size, reproduction function such

as fertility rate, or internal biomarkers such as key protein expression level, as the results of EDC exposure. For example, the expression of vitellogenin, an egg yolk precursor protein, was widely used as the indicator of estrogenic response in male fish models including zebrafish, sheephead minnow, madaka etc.(Folmar, Hemmer et al. 2000, Rose, Holbech et al. 2002, Kurauchi, Nakaguchi et al. 2005). For evaluation of EDCs' effects on mammals, mouse models provided several different assays to evaluate interference of estrogen, androgen, steroidgenesis and thyroid related functions(Gray, Wilson et al. 2004). For example, ovariectomized mouse based uterotropic assay (measure the weight change of uterus), and pubertal female assay (measure early opening of vagina), were commonly used to determine the estrogenic activity of EDCs(Kim, Shin et al. 2002, Ohta, Takagi et al. 2012, Hewitt, Winuthayanon et al. 2015). Hershberger assay and pubertal male assay (both measure weight change of androgen-related organs) were used to determine EDCs affecting androgen and thyroid related functions(Gray, Furr et al. 2001, Marty, Crissman et al. 2001). The main advantage of *in vivo* assays is that test results represent the actual reaction of the organism to the exposure of tested EDCs. As the endocrine system involves complex pathways, it is necessary to incorporate *in vivo* assays in order to properly evaluate the systematic reaction of the entire endocrine system. *In vivo* assays are also particularly suitable for evaluating the effect of exposure to a mixture of EDCs, as well as long-term environmental exposure evaluation.

Compared to *in vivo* assays, *in vitro* assays can be performed much quicker with lower cost, therefore are very suitable for large scale screening while still being able to provide biologically relevant EDCs potency evaluation. Most *in vitro* assays were designed to examine the ability of EDCs to interact with the targeted receptor protein (i.e. estrogen receptor). For example, ligand

identification with estrogen receptor has been successfully developed in the cell free form such as enzyme-linked receptor assay (ELRA), as well as a commercially available fluorescent receptor-binding assay etc.(Seifert, Haindl et al. 1999, Wittliff, Andres et al. 2008). Cell based *in vitro* assays typically examines the activation of hormonal receptors by EDCs, and generate the response signal through receptor-dependent expression of reporter genes. Since the 1990s, a variety of cell based bioreporter assays were developed using both yeast and mammalian cell lines(Leusch, de Jager et al. 2010). These bioreporters express hormonal receptors such as estrogen/androgen receptor (endogenous or artificially introduced), and harbor reporter gene expression cassette that is controlled by the hormone response element, a short sequence of DNA within the promoter that directly interacts with the hormone receptor complex. While the fundamental mechanism of these reporters are similar, selected reporter genes varied from β-galactosidase, green fluorescent protein to different type of luciferases (Routledge and Sumpter 1996, Bovee, Helsdingen et al. 2004, Sanseverino, Eldridge et al. 2009).

Among the different type of bioreporters, yeast bioluminescence reporters *Saccharomyces cerevisiae* BLYES, BLYAS and BLYR were developed with the aim of being a high-throughput, sensitive, rapid and low-cost EDCs screening tool (Sanseverino, Gupta et al. 2005, Eldridge, Sanseverino et al. 2007). BLYES, BLYAS and BLYR were designed as fully autonomous bioluminescent strains to detect estrogenic, androgenic or toxic compounds respectively. BLYES and BLYAS were developed on the basis of two *S. cerevisiae* strains expressing chromosomally maintained human estrogen receptor alpha and human androgen receptor gene respectively(Purvis, Chotai et al. 1991, Routledge and Sumpter 1996). These strains were further engineered to carry the bioluminescence gene cassette *luxCDABE* from *Photorhabdus*

luminescens, and the expression of *lux* genes are either constitutive for cytotoxicity detection (BLYR), or controlled by estrogen response element (BLYES) (Fig 1.3), and androgen response element (BLYAS). Upon exposure to estrogenic or androgenic compounds, BLYES and BLYAS can provide quantitative measurement through dose-dependent bioluminescence production. BLYR can provide cytotoxicity evaluation through bioluminescence reduction. The yeast bioluminescent reporters strains have been successfully used in various applications including the screening of 71 chemicals for hormonal and toxic effects(Sanseverino, Eldridge et al. 2009), surface and drinking water estrogenicity evaluation in Brazil(Di Dea Bergamasco, Eldridge et al. 2011), estrogenicity/androgenicity evaluation for plastic monomer and biotransformed EDCs(Lee, Zhao et al. 2012, Osimitz, Eldridge et al. 2012, Krifaton, Kriszt et al. 2013), estrogenicity evaluation in municipal sewage and sludge(Ruan, Liang et al. 2015, Wang, Eldridge et al. 2015). Compared to mass-based chemical analysis, yeast bioluminescent assays can provide biologically relevant estimation on the cumulative effect of hormonally active compounds in environmental samples without having to identify the individual compound. Additionally, yeast bioluminescent reporters have comparable detection sensitivity as chemical analysis, respond quickly (≤ 4 hours), and can test samples in high-throughput at much lower costs, resulting in a suitable screening tool for wastewater EDCs that can be combined with chemical analysis when more targeted analysis is required.

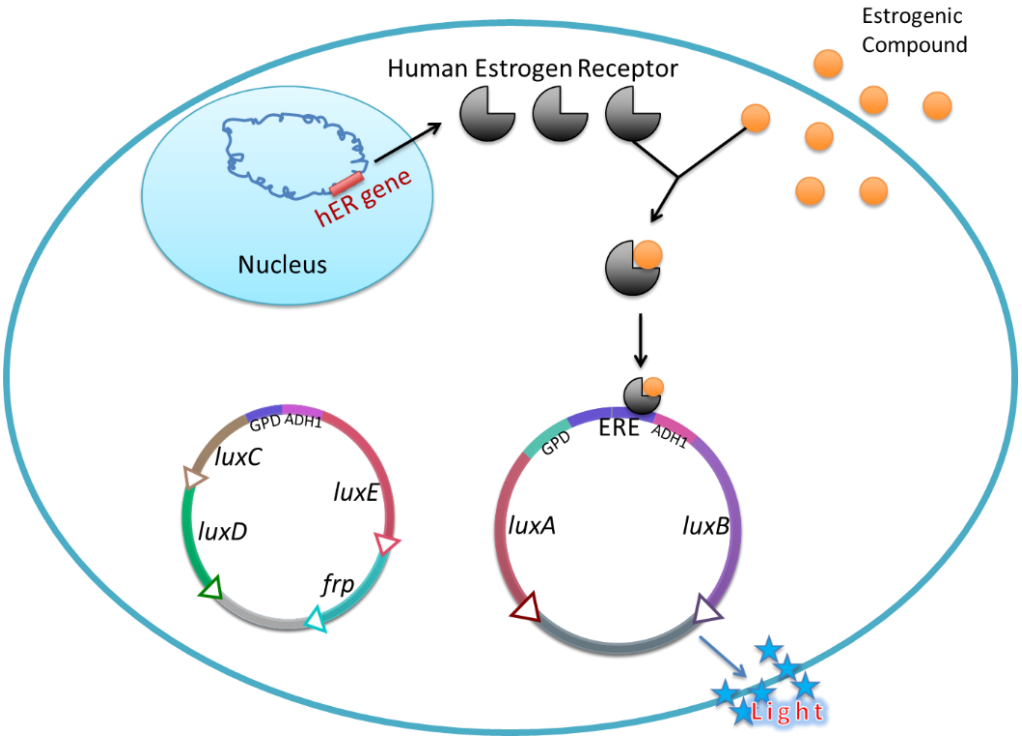

Fig 1.3. Schematic of bioluminescent yeast estrogen reporter. Human estrogen receptor protein was chromosomally integrated and expressed in the yeast. When estrogen receptor is activated by estrogenic compound, it binds to the estrogen response element (ERE) sequence on the plasmid, triggering the expression of the plasmid to produce luciferase and light.

1.2 Human Estrogen Receptor Protein

Structure of ER

Human estrogen receptors (hER) are members of the nuclear receptor superfamily that mediates estrogen regulated physiological and reproducible functions as transcriptional factors. So far two hER genes, ESR1 and ESR2, have been identified in human, with each coded protein referred as ERα and ERβ in the order of discovery(Thomas and Gustafsson 2011). Similar to other members of the nuclear receptor family, both ERα and ERβ protein have 6 functional domains (Fig 1.4): the N-terminal A and B domain, which contain the ligand-independent activation function 1 (AF-1), DNA binding domain (DBD), which is responsible to bind to DNA sequence referred as estrogen response element (ERE), a flexible hinge domain, and a C-terminal ligand binding domain (LBD) that contains ligand-dependent activation function 2 (AF-2). The full length ERα and ERβ are 595 and 530 amino acids respectively. ERα and ERβ share very high homology in the DNA binding domain (96%), moderate homology in the ligand binding domain (53%), and very low in A/B domain (30%). ERα and ERβ expression has different tissue distribution, and their individual function still remains to be cleared, though in breast cancer, ERα is expressed in ~70% of tumors with significantly higher level than ERβ(Böttner, Thelen et al. 2014, Thomas and Gustafsson 2015). This present study solely focused on ERα.

Crystal structures are only available for the DBD and LBD of ER (Fig 1.5). The lack of whole ER protein crystal structure is partly due to intrinsic flexibility of the hinge domain that hinders the formation of crystal. The AF-1 domains lacks defined structure in solution but forms secondary structure when interacting with coregulators(Rajbhandari, Finn et al. 2012). The DBD is a

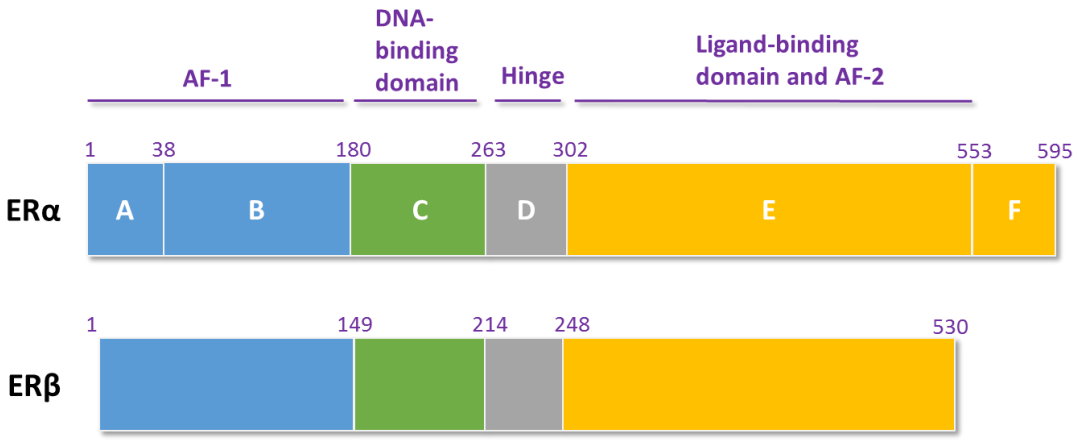

Fig 1.4. Schematic representation of the functional structure of estrogen receptor alpha and beta. The structural domains are labeled A-F and the number of amino acids are labeled in purple. The location of the functional domains are marked in purple bars. Region A and B contains the ligand-independent activation function 1 (AF1). Region C is the DNA binding domain responsible for binding to estrogen response element (ERE). Region D is the flexible hinge domain. Region E is ligand binding domain, contains the ligand-dependent activation function 2 (AF2).

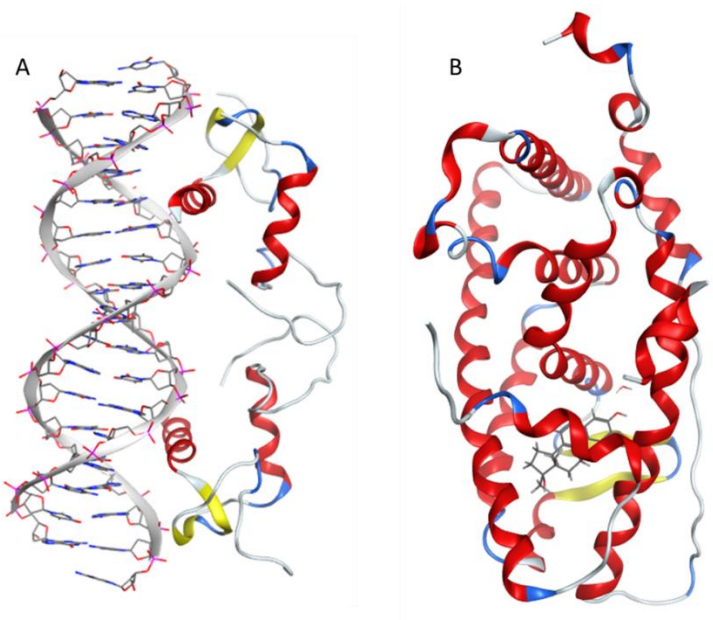

Fig 1.5. Crystal structure of estrogen receptor DNA binding domain (A) and ligand binding domain (B).

conserved domain in nuclear receptor, contains two zinc fingers that recognize the ERE sequence(Schwabe, Chapman et al. 1993). The C-terminal LBD is consisted of 12 alpha helix (H1-12) that forms a hydrophobic ligand binding cavity responsible for high affinity binding of estrogens. Besides ligand binding, the AF-2 region of the LBD is responsible to recruit various co-regulatory proteins that are required for ER's transcriptional activity. The LBD is also involved in receptor dimerization that is necessary for DBD to bind to the ERE sequence(Brzozowski, Pike et al. 1997).

Transcriptional activation by ER

As transcriptional factors, ERs' regulatory function can be either estrogen-dependent or independent. The estrogen-independent transcriptional activation function is fulfilled by the AF-1 domains, and the function can be controlled by phosphorylation through the MAPK pathway(Rajbhandari, Finn et al. 2012). The estrogen-dependent transcriptional regulation is the primary machinery for ER to respond to the serum level change of estrogens and mediate the regulatory functions. Upon binding of estrogens, ER undergoes conformational changes that further lead to receptor dimerization, binding to ERE sequence, recruitment of coregulator and other components in the transcriptional complex to regulate gene expression (Fig 1.6).

Agonist and antagonist ligand-receptor interaction

The binding pocket of the DBD has a flexible helix-12 that, depends on the type of ligand, the helix-12 can undergo an open or closed position (Fig 1.7). When agonist such as natural estrogen bind to the pocket, helix-12 can successfully close the binding pocket, allowing co-activator to bind to the protein. However an antagonist such as 4OH-tamoxifen has a long side chain that

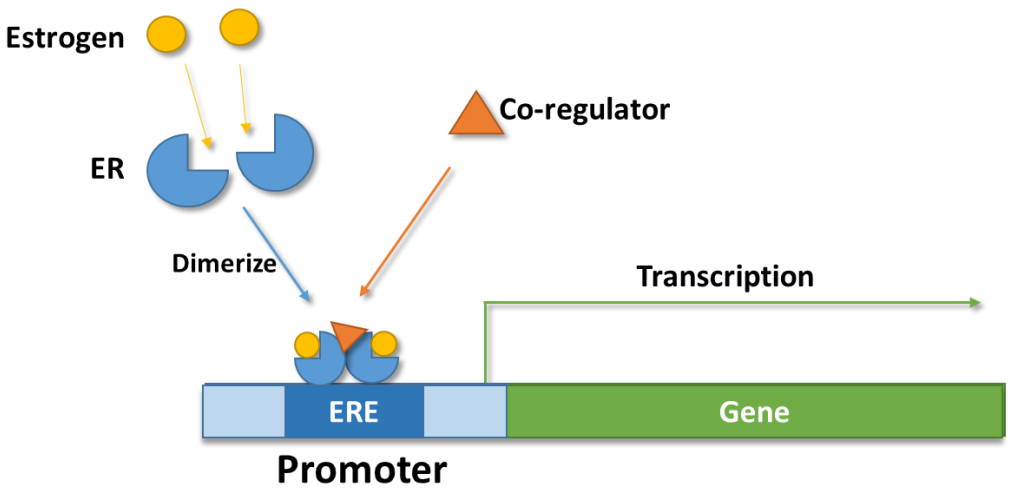

Fig 1.6. Schematic of estrogen-dependent transcriptional regulation by estrogen receptor. Ligand binding activates the ER to dimerize, bind to estrogen response element (ERE) sequence within a promoter, recruit co-activators and other components in the transcriptional complex.

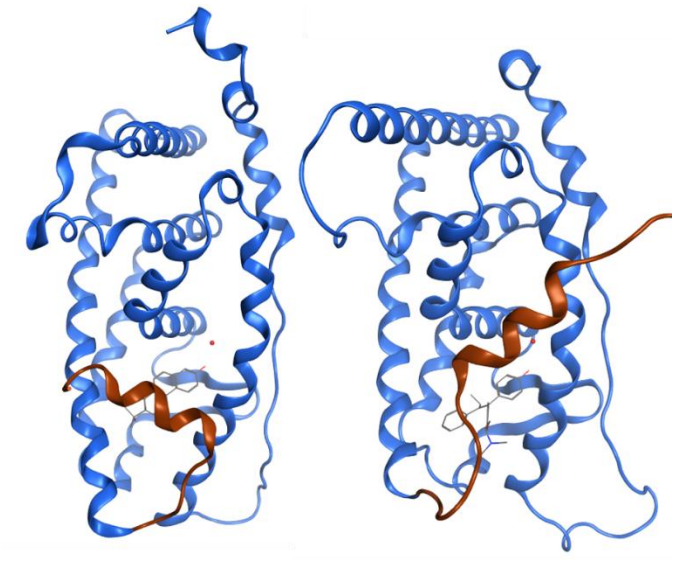

Fig 1.7. Agonist-bound form and antagonist-bound form of estrogen receptor ligand binding domain. When bound by agonist, helix-12 (marked in brown) is in the closed position, while antagonist binding will disturb the positioning of helix-12 to close the binding pocket.

perturb the positioning of helix-12, rendering an open position that disrupt the co-activator binding surface(Brzozowski, Pike et al. 1997, Shiau, Barstad et al. 1998). The ligand binding pocket of ERα consists of mostly hydrophobic amino acids with several charged residues providing critical electrostatic interaction with ligands (Fig 1.8). When bound by natural estrogen 17β-estradiol, the charged side chain of Glu353 and Arg394, along with a water molecule form a hydrogen network that interacts with the phenolic hydroxyl group of the ligand. On the other end of the ligand, His524 forms a hydrogen bond with the hydroxyl group of the ligand as well. Other hydrophobic residues further shape the binding cavity, providing a hydrophobic "clamp" to properly position the ligand. Due to the flexibility of ER ligand bind pocket, many natural and artificial compounds other than natural estrogens can also bind to the DBD and exert either agonist or antagonist effect. This is the main reason why many structurally diverse EDCs can interact with estrogen receptor.

ER mutations and human disease

As ER plays vital role in human physiology and reproduction, there has been considerable interest in identifying and associating mutations in ER with human diseases. However, since the finding of the first missense naturally occurring ERα mutation (Ala86Val) in breast cancer in 1988, natural mutations in ERα are rarely reported in primary diseases(Garcia, Lehrer et al. 1988). The reported polymorphisms in the ERα gene that alter the protein sequence are predominantly related to breast cancer tissues, though other health problem including endometrial cancer, physiological and developmental disorder were also involved(Herynk and Fuqua 2004, Li, Liu et al. 2005, Quaynor, Stradtman et al. 2013). Some notable mutations include Ala86Val located at the AF-1 domains, Lys303Arg located at border of the hinge and LBD, Tyr537Ser located at the LBD etc.

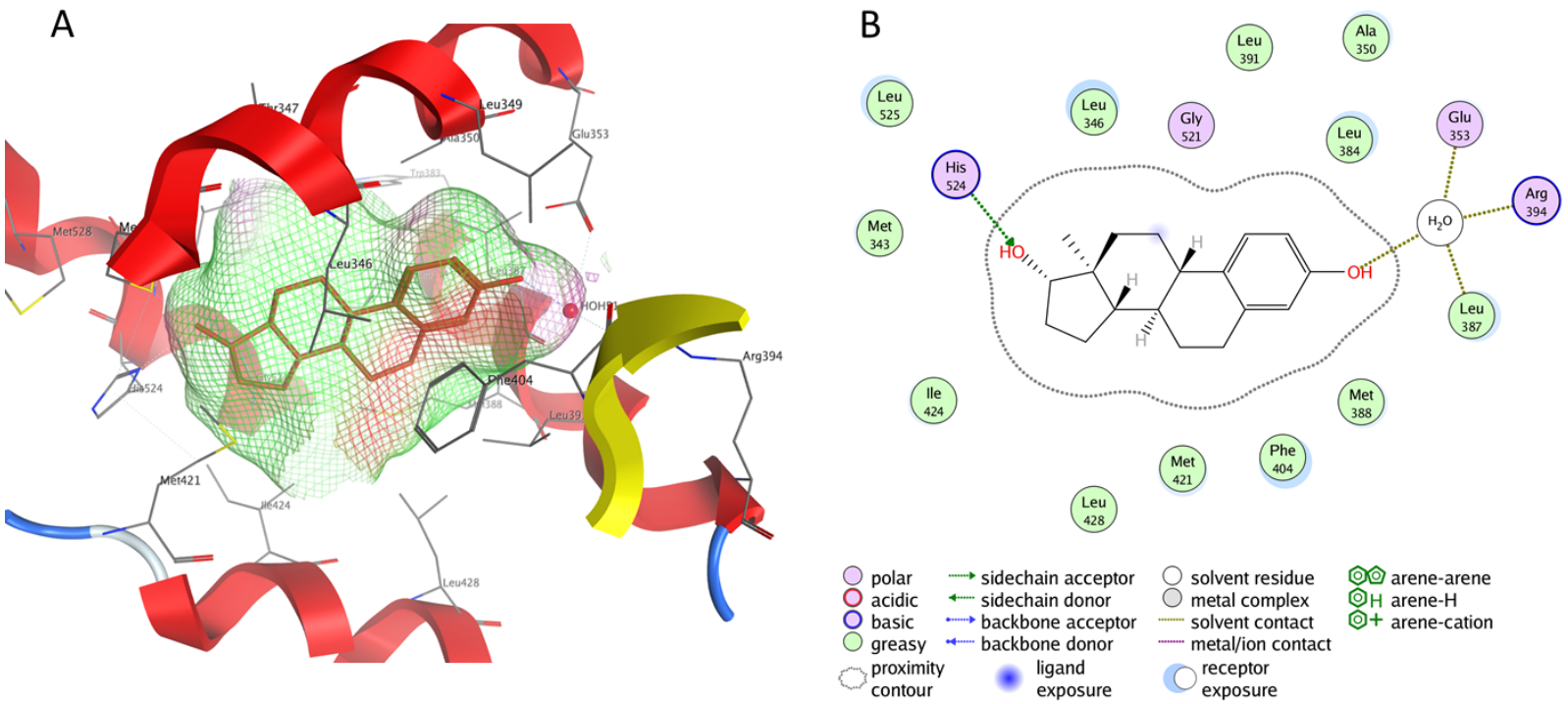

Fig 1.8. Natural estrogen 17β-estradiol in hER alpha binding pocket. (A) 3D demonstration of the ligand in the pocket (PDB entry: 1A52). Binding pocket surface is represented in the net surface, with green representing a hydrophobic surface, purple and red representing polar surfaces. (B) 2D schematic of the ligand-protein interaction. Glu353 and Arg394 provide critical electrostatic interaction (forming a hydrogen bond network with a water molecule) with the phenolic hydroxyl group of the ligand. These two residues are necessary for the ligand to properly position in the binding pocket. On the other side of the binding pocket, His524 provide critical hydrogen bond interaction with the ligand as well. The rest of the residues provide hydrophobic environment on the two sides of the ligand, forming a hydrophobic “clamp” around the ligand.

The situation of the limited ER mutant reports in the literature was changed since 2013, when several groups revealed numerous mutations of ERα in metastatic breast cancer tissues, with some occuring at much higher high frequency than previously known(Merenbakh-Lamin, Ben-Baruch et al. 2013, Robinson, Wu et al. 2013, Toy, Shen et al. 2013, Jeselsohn, Yelensky et al. 2014). These studies find that breast cancer patients can acquire resistance to anti-estrogen therapy as the result of a few point mutations occurred at Tyr537 and Asp538 on Helix-12 of the LBD. The mutations at Tyr537 and Asp538 can induce Helix-12 to stay in the closed position, making the ER constitutively active and subsequently promote the proliferation of tumor cells, thus making anti-estrogen drug such as tamoxifen ineffective(Thomas and Gustafsson 2015). When collecting all the recent clinical screening studies on ESR1 gene, it is clear that the majority of mutations occur at the ligand binding domain (Fig 1.9). When analyzing these clinical studies together, the mutation rate in ERα in primary tissues was only about 1% among all the patients, while mutation frequency in metastatic tissue reached around 19%(Segal and Dowsett 2014). These recent studies provided substantial evidence that mutations naturally occurring on the LBD of ERα are directly involved in the development of breast cancer, and their roles in interfering the interaction between the ER and its ligands, be it the natural estrogen, anti-hormone drugs or estrogenic EDCs, require further in-depth investigation to allow better control of ER-involved cancers.

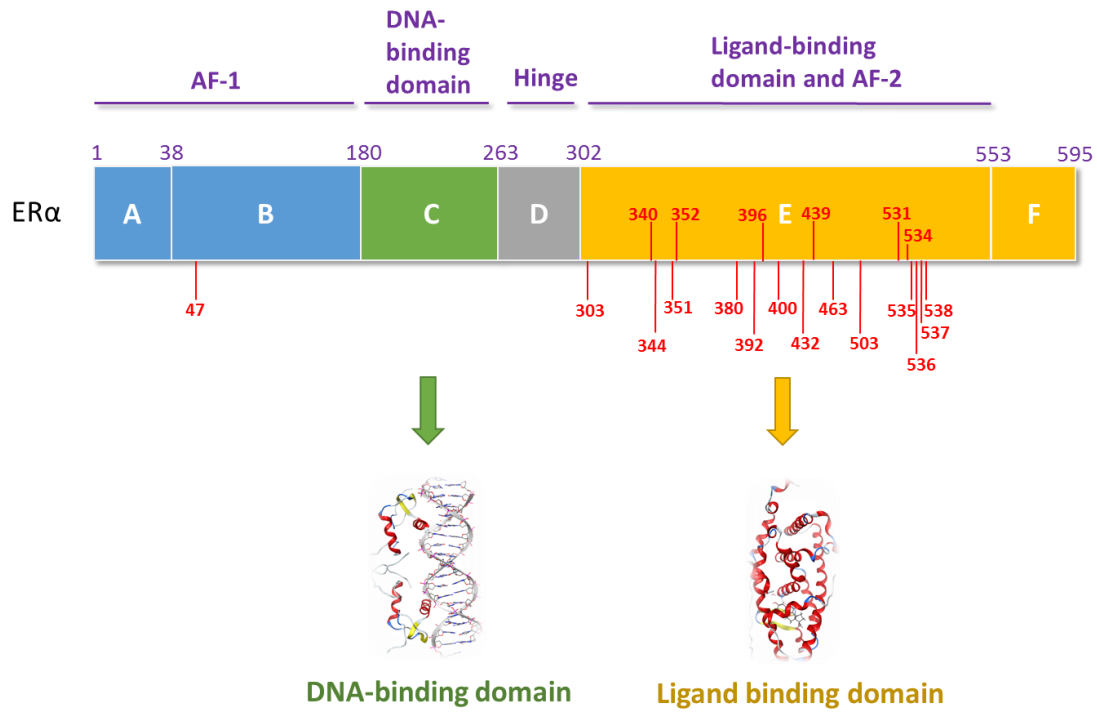

Fig 1.9. Schematic of human estrogen receptor alpha functional domains and natural mutations found in breast cancer. Mutations found in primary and metastatic breast cancer tumors reported in previous studies(Roodi, Bailey et al. 1995, Segal and Dowsett 2014, Thomas and Gustafsson 2015) are marked in red. The structural domains are labeled A-F and the number of amino acids are labeled in purple. The location of the functional domains are marked in purple bars. Region A and B contains the ligand-independent activation function 1 (AF1). Region C is the DNA binding domain responsible for binding to estrogen response element (ERE). Region D is the flexible hinge domain. Region E is ligand binding domain, contains the ligand-dependent activation function 2 (AF2).

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Chapter 2. Assessing Microbial Modulation of EDC Potential in Wastewater Treatment Using Yeast Bioluminescent Reporters

A version of this chapter was originally published by Jun Wang, Melanie Eldridge, Fu-min Menn, Todd Dykes, Gary Sayler:
Jun Wang, Melanie Eldridge, Fu-min Menn, Todd Dykes, Gary Sayler. "Standardized application of yeast bioluminescent reporters as endocrine disruptor screen for comparative analysis of wastewater effluents from membrane bioreactor and traditional activated sludge" 2015. Ecotoxicology
The work and writing presented in this paper was done by Jun Wang.

Abstract

A standardized protocol is demonstrated for bioluminescent strains *Saccharomyces cerevisiae* BLYES, BLYAS and BLYR as high-throughput screening tools to monitor the estrogenic, androgenic and toxic potencies in wastewater. The sensitivity and reproducibility of the assay in wastewater monitoring was evaluated for 7 day semi-continuous batch reactor using activated sludge with hormones spiked raw sewage. Yeast bioluminescent assay successfully captured the rapid removal of estrogenic and androgenic activities in the bioreactors, and demonstrated rapid response (≤ 4 hrs) with good reproducibility. This standardized protocol was then applied in a 12 months monitoring of the effluent of a WWTP located at Powell, TN, USA featuring parallel-operated full-scale membrane bioreactor (MBR) and traditional activated sludge (TAS) treatment. Monitoring results showed that estrogenic activity was persistent in all TAS and most MBR effluent samples, while residual androgenic activity was non-detectable throughout the monitored period. The estrogenic equivalents (EEQ) in TAS effluent ranged from 21.61 ng/L to 0.04 pg/L and averaged 3.25 ng/L. The EEQ in MBR effluent ranged from 2.88 ng/L to 0.0134 pg/L and averaged ~10 fold less (0.32 ng/L) than TAS. Despite the large temporal variation, MBR effluent EEQ was consistently lower than TAS on any given sampling date. Most MBR effluent samples also exhibited less cytotoxicity than TAS. Further analysis did not demonstrate significant

correlation between effluent EEQ level and WWTP operational parameters including MLSS, SRT, HRT and BOD.

2.1 Introduction

Endocrine disruptors (EDCs) refer to natural and synthetic chemicals that can cause a variety of adverse health effects in human and animals by interfering with the function of endocrine system. Commonly studied EDCs included natural hormones such as 17β-estradiol and dihydrotestosterone, synthetic hormones such as 17α-ethinyl estradiol, phytoestrogens such as genistein, and industrial materials such as bisphenol A or nonylphenol etc(Liu et al. 2009). Since the potential health risk of EDCs was initially discovered, EDCs are implicated in numerous hormone related problems such as reduced reproducibility, birth defects, abnormal development, osteoporosis, breast cancer, etc

(Frye et al. 2012; Kabir et al. 2015). Due to human activities and waste discharge, EDCs are suggested as a cause of reproductive abnormities in wildlife, and have been detected in surface, ground and drinking water around the world (Kim et al. 2007; Kolpin et al. 2002; Luo et al. 2014).

One of the major sources of environmental EDCs is industrial and municipal sewage. As the primary barrier preventing the release of EDCs into the aquatic environment, most wastewater treatment plants (WWTP) rely on concentrated microbial biomass, activated sludge, to metabolize and remove organic pollutants including EDCs. However the commonly used activated sludge in WWTP isn't specifically designed for trace organic chemical removal and residual EDCs are continuously discharged from WWTPs at various concentrations (Liu et al. 2009). As the concern over the health and ecological risk of environmental EDCs persists, much

research has focused on improving the efficiency of EDC removal in wastewater treatment and improving monitoring protocols. Apart from optimizing WWTP operational parameters such as mixed liquor suspended solid (MLSS) concentration, solid retention time (SRT), hydraulic retention time (HRT) etc, new secondary or tertiary treatment technologies such as membrane bioreactors, ozonation and advanced oxidation processes, activated carbon adsorption etc. were developed and studied for their EDCs removal efficiency(Chang et al. 2009; Luo et al. 2014). Since EDCs removal efficiency can be affected by the treatment technologies as well as the properties and contents of the wastewater, effective screening tools are required to monitor and evaluate the performance of WWTPs. During wastewater treatment, reduction of a parent compound doesn't necessarily result in reduced biological activity as the biotransformed products could still have potent or even augmented hormonal activity. Therefore when assessing the EDCs removal efficacy in wastewater treatment, besides tracking the fate of the targeted compound, it is necessary to evaluate the changes in biological activities as well.

In the need of effective EDCs evaluation in wastewater treatment, yeast bioluminescence reporters *Saccharomyces cerevisiae* BLYES, BLYAS and BLYR have shown great potential of being a high-throughput, sensitive, rapid and low-cost EDCs screening tool (Eldridge et al. 2007; Sanseverino et al. 2005). BLYES, BLYAS and BLYR were previously developed as fully autonomous bioluminescent strains to detect estrogenic, androgenic or toxic compounds respectively. BLYES and BLYAS were developed on the basis of two *S. cerevisiae* strains expressing chromosomally maintained human estrogen receptor alpha and human androgen receptor gene respectively(Purvis et al. 1991; Routledge and Sumpter 1996). These strains were further engineered to carry the bioluminescence gene cassette *luxCDABE* from *Photorhabdus*

luminescens, and the expression of *lux* genes are either constitutive for cytotoxicity detection (BLYR), or controlled by estrogen response element (BLYES) and androgen response element (BLYAS). Upon exposure to estrogenic or androgenic compounds, BLYES and BLYAS can provide quantitative measurement through dose-dependent bioluminescence production. BLYR can provide cytotoxicity evaluation through bioluminescence reduction. The yeast bioluminescent reporters strains have been successfully used in various applications including the screening of 71 chemicals for hormonal and toxic effects(Sanseverino et al. 2009), surface and drinking water estrogenicity evaluation in Brazil(Di Dea Bergamasco et al. 2011), estrogenicity/androgenicity evaluation for plastic monomer and biotransformed EDCs(Krifaton et al. 2013; Lee et al. 2012; Osimitz et al. 2012), estrogenicity evaluation of bisphenol analogs in municipal sewage sludge(Ruan et al. 2015). Compared to mass-based chemical analysis, yeast bioluminescent assays can provide biologically relevant estimation on the cumulative effect of hormonally active compounds in environmental samples without having to identify the individual compound. Additionally, yeast bioluminescent reporters have comparable detection sensitivity as chemical analysis, respond quickly (≤ 4 hours), and can test samples in high-throughput at much lower costs, resulting in a suitable screening tool for wastewater EDCs that can be combined with chemical analysis when more targeted analysis is required.

The main objectives of the present study are: (a) to present a standardized protocol using yeast bioluminescent reporter assay for high-throughput wastewater EDC screening, (b) to demonstrate the sensitivity, reproducibility of the assay in wastewater monitoring, and (c) to apply the yeast bioluminescent reporters in a long-term comparative study on parallel-operated full-scale membrane bioreactor (MBR) and traditional activated sludge (TAS). Lab-based semi-

continuous bioreactors were initially used to test the sensitivity and reproducibility of the standardized yeast assay in capturing the rapid change of hormonal activities in the bioreactors. Then the assay was employed in a 12 months monitoring of effluent from parallel-operated full-scale TAS and MBR located at Powell, TN, USA. In prior studies, MBR and TAS were compared either on lab or pilot scale plants, or were compared on different locations (Clara et al. 2005b; Kimura et al. 2007; Vega-Morales et al. 2013). This study provides the first long-term performance assessment and comparison on the EDCs discharge at a full-scale WWTP with parallel-operated MBR and TAS. The androgenic, estrogenic and toxic activities in monitored MBR and TAS effluent were reported and their correlation with key operational parameters were analyzed. The complete wastewater testing protocol including sample preparation, bioassay setup, and data analysis is described in full detail. The issue of wastewater toxicity in yeast bioassay, estrogenic equivalent calculation and result interpretation are also discussed.

2.2 Materials and Methods

Chemicals

Natural estrogen 17β-estradiol (E2, CAS# 50-28-2), synthetic estrogen ethinyl estradiol (EE2, CAS# 57-63-6) and natural androgen 5α-dihydrotestosterone (DHT, CAS# 521-18-6) were purchased form Sigma-Aldrich. High performance liquid chromatography (HPLC) grade methanol, acetone and acetonitrile were purchased from Fisher Scientific.

Sampling site

Wastewater influent, effluent and activated sludge samples were taken from Hallsdale-Powell Utility District (HPUD) Beaver Creek Wastewater Treatment Facility at Powell, Tennessee, USA.

This plant serves approximately 22,000 customer accounts covering ~260 km^2. The maximum wastewater processing capacity of the entire plant is ~ 45,000 m^3 per day. The wastewater influent of this plant consists mostly sanitary domestic sewage, as well as three major industrial users including a bread baking operation, a milk processing facility and a meat repackager. The plant operates with two parallel facilities including a traditional activated sludge (TAS) coupled with secondary clarifiers, and a membrane bioreactor (MBR) featuring immersed GE ZeeWeed-500 (originally ZENON, Oakville, Ontario) Hollow-Fiber membranes. Raw wastewater influent is pretreated by coarse screening, grit and grease removal, and then separated into either TAS or MBR feed to be treated. Effluents from TAS and MBR are discharged together into Beaver Creek. Operational parameters of the wastewater facility were monitored and provided by HPUD for this study. Parameters monitored separately for TAS and MBR included MLSS, MLVSS, solid retention time (SRT), hydraulic retention time (HRT), food/microbe ratio (F/M ratio). Other operational parameters monitored for the influent and effluent of the entire plant and included the total flow, carbonaceous 5-day biological oxygen demand ($cBOD_5$), suspended and settled solid, dissolved oxygen and ammonia content.

Semi-continuous fill & draw lab-scale bioreactor experiment

Activated sludge and raw wastewater were sampled from HPUD in pre-baked (500 °C for 4 hours) bottles and transported in icebox to the lab. Raw wastewater was immediately filter (0.22 μm) sterilized and kept in dark at 4 °C before feeding the bioreactors. Fill & draw bioreactor experiments were started on the same day of sampling. Two experiments (Table 2.1) was performed using the activated sludge from the TAS and MBR respectively. At the time of sampling, MLSS in TAS was ~2500 mg/L, while the MLSS in MBR was ~8500 mg/L. Since the biosolid

concentration in the MBR is higher than that in the TAS, the spiked chemical concentrations were increased 10 fold in the second experiment.

In each experiment, 3 groups of 100 mL bioreactors in triplicate were set up in the lab (Fig. 2.1), including (1) activated sludge (AS), (2) activated sludge with spiked hormones (AS+), (3) filtered raw wastewater (- control). Bioreactors were maintained at 28 °C, 200 rpm and the fill & draw operations continued for 7 days. In each day 30 mL mixed liquid were taken out of each bioreactor and re-filled with filtered raw wastewater (group 2 were also spiked with hormones). Collected samples were centrifuged for 10 minutes at 5000 rpm to separate the liquid and solid phase. The separated solid phases in the 2nd fill & draw experiments were dried at 103 °C for 3 hours to measure the MLSS. Liquid phases were extracted using solid phase extraction (Oasis HLB 20cc cartridge, Waters, USA) following the US EPA method 1694 and eluted in distilled methanol. Eluates were dried by high purity nitrogen gas and preserved in dark at -20 °C. Prior to yeast bioluminescence assay, extracts were re-dissolved in 0.75mL distilled methanol (40X concentrates).

TAS and MBR wastewater effluent sampling and extraction

Field wastewater effluents from TAS and MBR at Hallsdale-Powell wastewater facility were sampled weekly from June 2011 for the first 14 weeks and then sampled less frequently until June 2012, spanning 12 months with a total of 31 sampling dates (Table 2.2). Wastewater effluents treated by TAS and MBR were collected separately in pre-baked (500 °C for 4 hours) bottles and transported in icebox. Each time 1 L of MBR and TAS effluents were extracted and tested except for the last three sampling dates, effluents were sampled and tested in triplicate

Table 2.1. Parameters for lab-scale fill & draw batch reactor experiments

	Experiment 1 (TAS biomass)	Experiment 2 (MBR biomass)
Biomass Sampling Date	May 2011	July 2011
Total Suspended Solid	~2500 mg/L	~8500 mg/L
Spiked Estrogen (final concentration)	E2 (27.24 mg/L), EE2 (2.96 mg/L)	E2 (272.38 mg/L),
Spiked Androgen(final concentration)	DHT (29.04 mg/L)	DHT (290.44 mg/L)
Sample Collection Time	0, 1, 3, 7 day	0, 1, 2, 3, 7 day

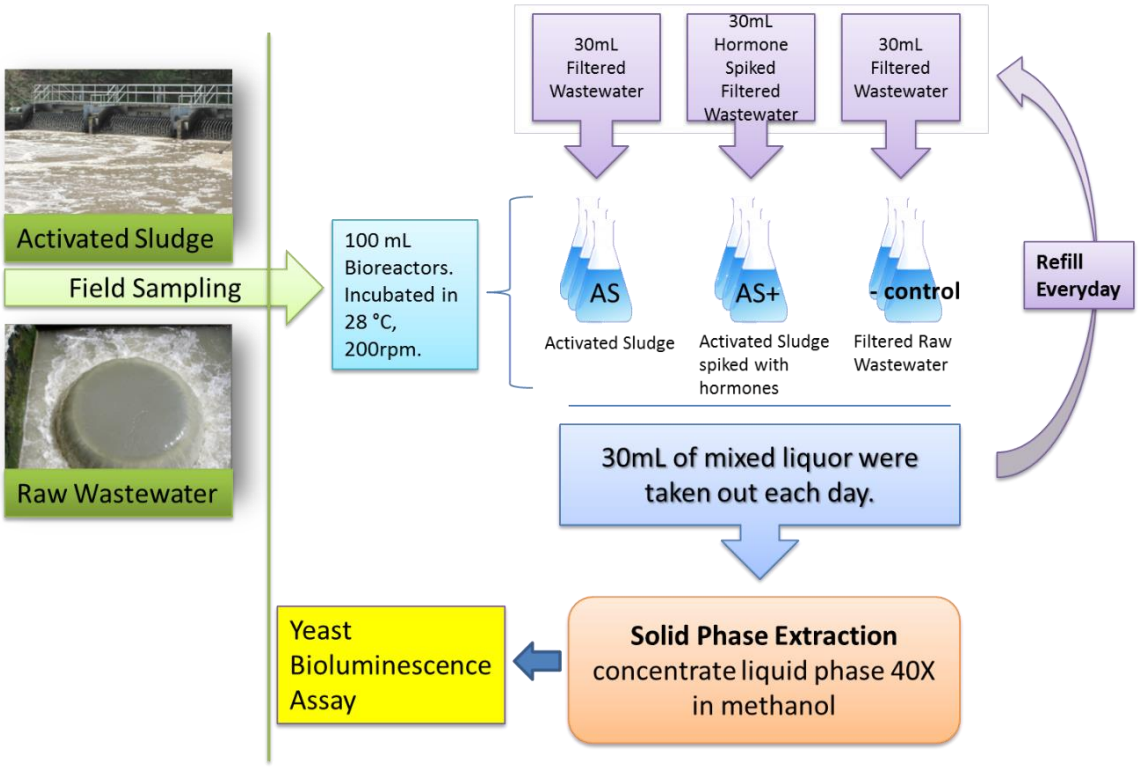


Fig 2.1. Schematic of fill & draw experiment. Three groups of 100 mL bioreactors including (1) activated sludge (AS), (2) activated sludge with spiked hormones (AS+), (3) filtered raw wastewater (-control) were maintained at 28 °C, 200 rpm. The Fill & Draw operations were conducted continuously for 7 days. In each day 30 mL mixed liquid samples were taken out of each bioreactor and re-filled with filter sterilized raw wastewater (group 2 were also spiked with hormones). Water samples were concentrated by solid phase extraction and tested by yeast bioluminescent assay.

to evaluate the variation in effluents from the same day. All water samples were extracted within 8 hours after sampling using modified US EPA Method 1694. Briefly, solid phase extraction disk (Oasis HLB disk, Waters, USA) was pre-soaked with 5 mL methanol for 5 min and then conditioned with 10 mL methanol followed by 10 mL distilled water with vacuum. Then 1 L water sample was acidified to pH 2.0 ± 0.5 with HCl and passed through the disk at < 40 mL/min under vacuum. After 15 min of air drying under vacuum, the SPE disk was soaked in 5 mL methanol for 2 min and eluted under vacuum. Then another elution was performed with 6 mL of 50:50 MeOH/Acetone and the collected eluate was dried by high purity nitrogen flow and stored at -20 °C. Extracts were re-dissolved in 1 mL distilled methanol prior to yeast bioluminescent assay (1000X concentrates).

To evaluate the recovery efficiency of the sample extraction method, a triplicate of 1L distilled water with 2×10^{-5} mole of spiked E2 and DHT were concentrated using the same *procedure for wastewater samples. The concentration of recovered E2 and DHT was determined by HP 6890 Gas Chromatography with HP 5973 Mass Spectrometer in Selected Ion Monitoring (SIM) acquisition mode. Deca-Cl-Biphenyl was used as the internal standard and helium was the carrier gas. Phenomenex ZB-50 GC column with 250 °C initial temperature and 1.0 mL/min initial flow was used for E2 detection. Agilent J&W DB-5ms GC Column with 260 °C initial temperature and 1.0 mL/min initial flow was used for DHT detection.

Yeast bioluminescence bioreporter assay (BLYES, BLYAS, BLYR)

Bioluminescent *Saccharomyces cerevisiae* BLYES (estrogen reporter), BLYAS (androgen reporter), BLYR (cytotoxicity reporter) (Sanseverino et al. 2009) were inoculated from -80 °C freezer stocks and grown in modified yeast minimal medium without leucine and uracil (YMM leu^-,

ura$^-$)(Routledge and Sumpter 1996) overnight at 28 °C, 200 rpm to reach OD 0.8 to 1.0 prior to sample testing.

Concentrated fill & draw experiment samples (40X) were serial diluted in distilled methanol to 12 dilutions ranging from 40X to 0.01X fold to the original sample. Concentrated wastewater effluent water samples (1000x) were serial diluted to 12 dilutions ranging from 1000X to 2.5X fold. 50 µL diluted samples were placed into the appropriate wells of Costar 96-well black plates (#3915 by Corning, Inc. USA) as mapped in Fig. 2.2A. On each plate, serial diluted E2 (1E-6 M to 2.5E-12 M) or DHT (1E-5 M to 2.5E-11 M) in distilled methanol were used as standard chemicals and positive controls for BLYES and BLYAS assay respectively. Solvent blanks (cell+medium+solvent) and true blanks (cell+medium) were included as negative controls. Loaded 96 wells plates were kept in room temperature to allow the solvent methanol evaporate and dry-out. Then 200 µL of yeast culture was added into each well of the 96 well plates. Each sample was tested by BLYES, BLYAS and BLYR to evaluate estrogenicity, androgenicity and cytotoxicity respectively. Bioluminescence was measured after 4 hours of sample exposure in a Perkin-Elmer Victor2 Multilabel Counter with an integration time of 1 s/well.

Data Analysis

Bioluminescence (counts per second) readings were plotted against the log of its concentrations (M), generating sigmoidal curves for estrogen (BLYES) and androgen (BLYAS) assays (Fig. 2.2CD). The concentration used for water samples were the concentration factors. For standards (E2/DHT), half maximal effective concentration (EC_{50}) was determined at the mid-point bioluminescence ((max-min)/2 + min) on the linear portion of the sigmoidal curve (Fig. 2.3a). For

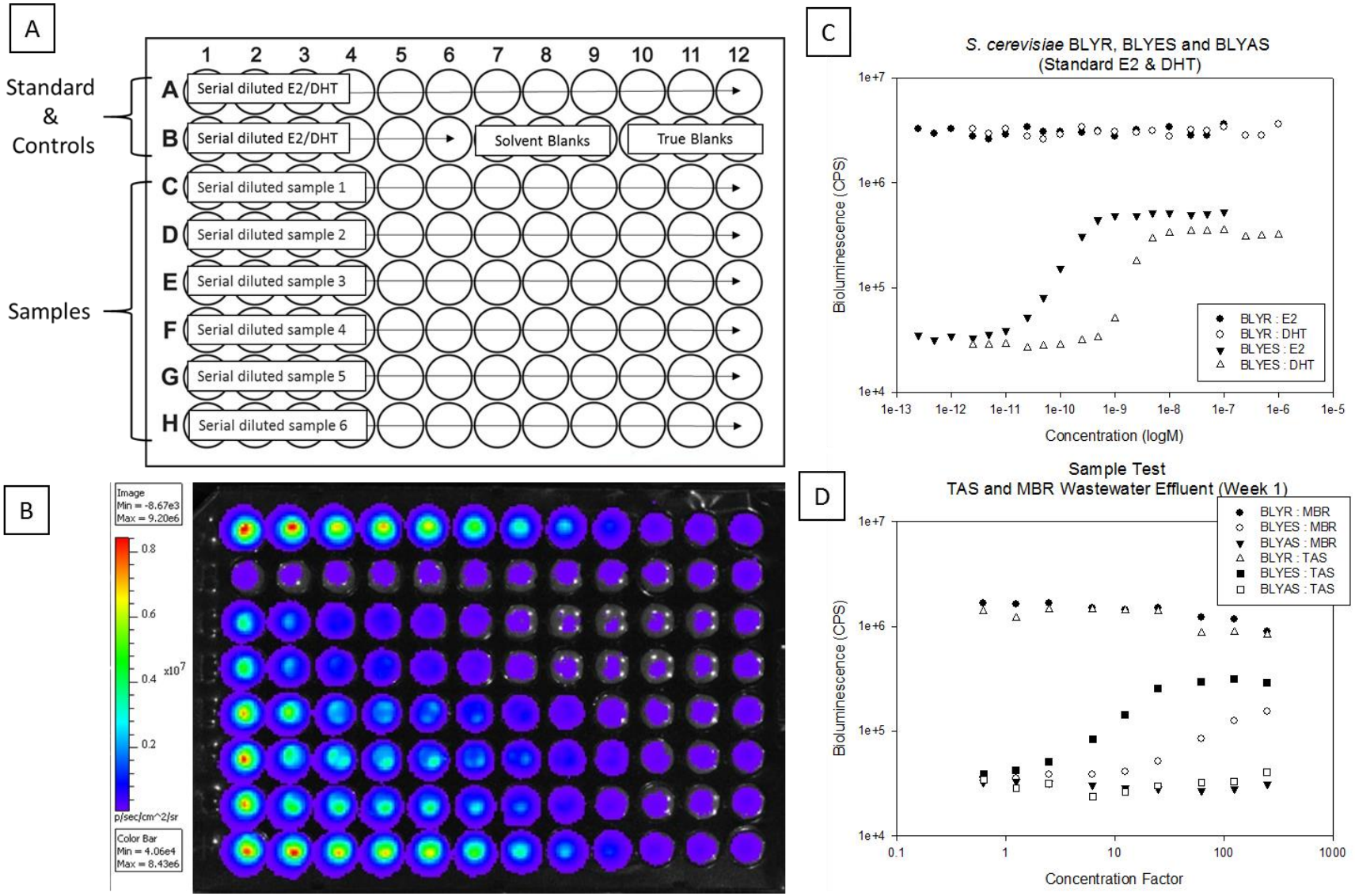
Table 2.2. Estrogenic Equivalent and Cytotoxicity of TAS and MBR effluent samples

Sampling Date		EEQ_{50} TAS (ng/L)	EEQ_{50} MBR (ng/L)	EEQ_{20} TAS (ng/L)	EEQ_{20} MBR (ng/L)	LDC TAS	LDC MBR	IC20 TAS	IC20 MBR
6/29/2011	Week 1	4.59E+00	2.39E-01	3.78E+00	2.98E-01	2.5	25	44.59	55.46
7/6/2011	Week 2	9.26E+00	2.08E+00	8.60E+00	2.28E+00	1.25	6.25	52.71	57.34
7/13/2011	Week 3	5.03E+00	3.09E-02	4.43E+00	8.68E-02	2.5	62.5	38.41	64.69
7/20/2011	Week 4	6.82E+00	2.88E+00	5.63E+00	2.49E+00	1.25	2.5	33.72	42.73
7/27/2011	Week 5	5.74E+00	3.67E-01	5.36E+00	1.20E-01	1.25	6.25	28.47	35.55
8/3/2011	Week 6	2.68E+00	2.81E-01	2.96E+00	5.73E-01	6.25	6.25	60.06	71.12
8/10/2011	Week 7	3.81E+00	3.57E-01	2.99E+00	5.62E-01	6.25	12.5	55.09	127.79
8/17/2011	Week 8	7.21E+00	2.65E-01	5.79E+00	4.72E-01	1.25	1.25	33.17	109.20
8/24/2011	Week 9	6.34E+00	3.37E-01	5.36E+00	4.74E-01	1.25	6.25	51.94	186.84
8/31/2011	Week 10	2.16E+01	4.53E-01	1.94E+01	4.54E-01	0.625	12.5	22.21	45.35
9/7/2011	Week 11	2.82E+00	3.55E-01	2.30E+00	4.31E-01	6.25	25	40.82	32.86
9/14/2011	Week 12	1.37E+00	6.46E-02	1.55E+00	1.29E-01	6.25	25	55.48	44.11
9/21/2011	Week 13	8.40E-01	1.04E-02	1.14E+00	3.60E-02	12.5	62.5	38.95	72.57
9/28/2011	Week 14	6.57E+00	4.30E-01	5.80E+00	4.23E-01	6.25	62.5	23.98	74.41
10/12/2011	Week 16	4.14E-01	7.42E-02	5.23E-01	1.11E-01	12.5	62.5	20.24	77.07
10/26/2011	Week 18	1.67E-01	2.40E-02	3.01E-01	4.19E-02	12.5	62.5	36.10	79.91
11/9/2011	Week 20	7.49E-01	4.42E-03	7.81E-01	1.98E-02	12.5	62.5	34.78	51.33
11/23/2011	Week 22	2.33E+00	3.30E-01	2.44E+00	2.81E-01	2.5	62.5	43.08	72.66
12/15/2011	Week 25	3.36E+00	1.65E-01	3.03E+00	1.75E-01	2.5	62.5	49.15	106.50
12/29/2011	Week 27	3.90E+00	1.84E-02	3.81E+00	4.56E-02	1.25	1.25	38.55	194.93
1/12/2012	Week 29	2.62E-01	3.92E-03	6.70E-01	9.34E-03	6.25	250	86.31	90.28
2/3/2011	Week 33	2.75E+00	2.22E-02	4.06E+00	3.00E-02	2.5	25	69.58	102.98
2/17/2011	Week 35	2.97E-02	3.22E-03	1.68E-01	3.25E-02	6.25	25	55.76	86.14
3/9/2012	Week 38	1.82E+00	6.56E-02	2.99E+00	3.69E-02	6.25	125	23.26	67.93
4/5/2012	Week 42	3.82E-05	nd	1.70E-03	nd	25	nd	19.73	17.28
4/25/2012	Week 45	3.04E-02	nd	1.33E-01	nd	12.5	nd	21.64	5.32
5/3/2012	Week 46	1.03E-01	2.13E-03	7.03E-01	2.24E-02	2.5	25	20.42	18.96
6/6/2012	Week 51	7.15E-02	2.36E-03	2.54E-01	2.86E-02	6.25	25	7.41	1.23
6/13/2012	Week 52	1.13E-02 (±1.96E-03)	1.34E-05 (±1.19E-05)	5.90E-02 (±5.77E-03)	4.58E-04 (±3.29E-04)	12.5	62.5	6.78	5.59
6/20/2012	Week 53	1.71E-02 (±1.33E-02)	2.33E-05 (±1.88E-05)	7.39E-02 (±2.90E-02)	6.42E-04 (±4.59E-04)	12.5	62.5	2.93	2.41
6/26/2012	Week 54	3.89E-04 (±5.36E-04)	nd	3.29E-03 (±3.99E-03)	nd	62.5	nd	2.05	1.15
	Average	3.25	0.32	3.07	0.35	8.21	43.93	36.05	64.57
	Std Dev	4.33	0.64	3.79	0.61	11.46	50.19	19.74	48.16

Nd: non-detectable

Table 2.2 Continued
EEQ50: estrogenic equivalents calculated by EC50 of 17β-estradiol and samples
EEQ20: estrogenic equivalents calculated by EC20 of 17β-estradiol and samples
LDC: lowest detectable concentration. The lowest concentration factor of tested sample that produces significant bioluminescence response (> 3 standard deviations of background) in BLYES assay.

Fig.2.2 Schematic of wastewater sample testing by S. cerevisiae BLYR, BLYES, and BLYAS on 96 well plates. (a). 96 well plate map for sample testing. In sample testing, each plate includes a serial diluted standard estrogen/androgen (E2 or DHT) to provide reference for sample estrogenicity and androgenicity estimation, as well as positive controls. Solvent blanks (methanol + cell culture) and true blanks (cell culture) are included as negative controls. In this layout, 6 serial diluted wastewater samples can be tested on each plate. Each sample is tested by BLYR, BLYES and BLYR on three separated plates respectively. (b). Bioluminescence production of *S. cerevisiae* BLYES after 4 hours exposure to standard E2 and wastewater samples on 96 well plate, captured by Xenogen IVIS Lumina Imaging System. Higher level of luminescence indicates stronger estrogenic response. (c). Standard dose-response curves of *S. cerevisiae* BLYR, BLYES and BLYAS after exposure to the E2 and DHT. Bioluminescence readings in the unit of counts per second (CPS) are plotted against log concentrations of the standard chemical. Constitutive light producing BLYR strain serves to monitor sample toxicity; a flat curve indicates no detectable cytotoxicity. BLYES and BLYAS produce sigmoidal curves upon exposure to E2 and DHT respectively. EC50 value for E2 and DHT can be calculated from the sigmoidal dose-response curves. (d). Wastewater sample (first week TAS and MBR effluent) testing results by *S. cerevisiae* BLYR, BLYES and BLYAS. Bioluminescence readings were plotted against log concentration factor of samples. The drop of BLYR curves at higher concentration indicate the presence of cytotoxicity in these sample dilutions.


A
Standard & Controls
Samples
1 2 3 4 5 6 7 8 9 10 11 12
A Serial diluted E2/DHT
B Serial diluted E2/DHT
Solvent Blanks
True Blanks
C Serial diluted sample 1
D Serial diluted sample 2
E Serial diluted sample 3
F Serial diluted sample 4
G Serial diluted sample 5
H Serial diluted sample 6
B
Image
Min = -8.67e3
Max = 9.20e6
0.8
0.6
0.4
x10^7
0.2
p/sec/cm^2/sr
Color Bar
Min = 4.06e4
Max = 8.43e6
C
S. cerevisiae BLYR, BLYES and BLYAS
(Standard E2 & DHT)
Bioluminescence (CPS)
1e+7
1e+6
1e+5
1e+4
1e-13 1e-12 1e-11 1e-10 1e-9 1e-8 1e-7 1e-6 1e-5
Concentration (logM)
BLYR : E2
BLYR : DHT
BLYES : E2
BLYES : DHT
D
Sample Test
TAS and MBR Wastewater Effluent (Week 1)
Bioluminescence (CPS)
1e+7
1e+6
1e+5
1e+4
0.1 1 10 100 1000
Concentration Factor
BLYR : MBR
BLYES : MBR
BLYAS : MBR
BLYR : TAS
BLYES : TAS
BLYAS : TAS

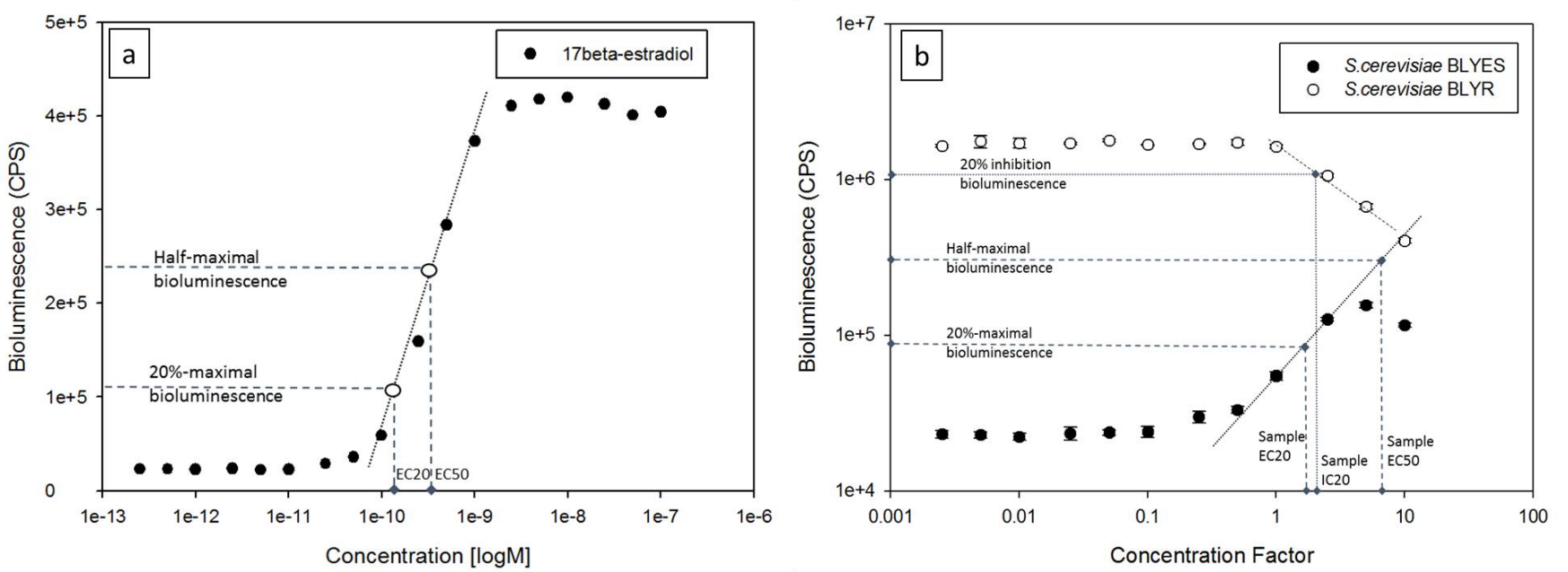


Fig.2.3 Comparative data analysis for yeast bioluminescent assays effective concentration calculation. (a) EC50 and EC20 calculation for standard chemical 17β-estradiol. (b) IC20, EC20, EC50 calculation for wastewater samples. (EC50 = half-maximal effective concentration, EC20 = 20%-maximal effective concentration, IC20 = 20%-maximal inhibitory concentration)

environmental samples, the concentration factor that yields half maximal bioluminescent response was used as the EC_{50} (Fig. 3b). Due to the cytotoxicity in concentrated environmental samples, sample testing may not be able to produce a full sigmoidal curve. Therefore linear regression was applied on the linear portion of data points and the obtained linear equation was used to calculate sample's EC_{50} using the half-maximal bioluminescence value obtained from the corresponding E2/DHT standard curve in the same batch of experiment. In all sample testing, a response was considered significant when the bioluminescence increase is higher than three times of the background bioluminescence standard deviation. Estrogenic equivalent (EEQ) and androgenic equivalent (AEQ) were calculated by using the EC_{50} of E2/DHT divided by the EC_{50} of each sample from the same batch of experiment. EEQ and AEQ represent the amount of hormonal active substances in the samples relative to E2/DHT. For wastewater effluents samples, EEQ was also calculated through the EC_{20} (20% maximal effective concentration) of the sample and standard, and was denoted as EEQ_{20}. To differentiate, EEQ calculated using EC50 was denoted EEQ_{50} in the result section. Cytotoxicity of wastewater samples tested by *S. cerevisiae* BLYR was reported as the concentration factor where 20% of bioluminescence inhibition (IC_{20}) occurred. To calculate IC_{20}, the data points showing inhibition were fitted with linear regression and the IC_{20} was inferred from the linear equation at 80% of the background bioluminescence.

2.3 Results and Discussion

E2 and DHT recovery efficiency by solid phase extraction

To evaluate the recovery efficiency of the wastewater extraction method, GC-MS was used to measure the recovery of natural estrogen (E2) and androgen (DHT) from spiked distilled water

(Table 2.3). E2 was determined to be an average of 4.45 ppm with 5.19% CV, and the recovery rate was averaging 81.71%. An average of 3.49 ppm DHT were detected in the concentrated sample with 46.27% coefficient of variation (CV), and an average of 60.05% recovery rate. The recovery rate for E2 and DHT were comparable to the recovery of tested chemicals in EPA's 1694 method report, though the CV of DHT recovery was slightly larger.

Total estrogenic and androgenic activity changes in fill & draw bioreactors

Lab-scale fill & draw bioreactors were used to simulate the removal process in wastewater treatment plant. The total estrogenicity, androgenicity and cytotoxicity in the effluent of fill & draw bioreactors were tested by *S. cerevisiae* BLYES, BLYAS and BLYR respectively. Fig 2.4 presents the plots of test results for the unspiked, spiked and negative control reactors from the two separate experiments. Calculated estrogenic equivalents and androgenic equivalents are presented in Table 2.4.

In the 1st fill & draw experiments (TAS), unspiked AS samples showed no detectable estrogenic response across all time points. The negative controls, which contained the filtered raw wastewater, showed weak response with average EEQ of 0.11, 0.02 and 0.04 ng/L at 0, 24 and 72 h respectively, validating the presence of estrogenic compounds in the raw wastewater. No estrogenic response were detected at 168 h, which may be due to an error in the last refill operation that yeast minimal media was mistakenly added into the reactors. In comparison, the spiked TAS bioreactor samples showed very strong estrogenic responses at time 0 due to spiked E2 and EE2. The calculated EEQ at time 0 was averaging 332.66 ng/L. At 24 hour, estrogenic response significantly decreased, and the EEQ reduced to an average of 1.64 ng/L. At 72 hours

Table 2.3. Recovery rate of natural estrogen and androgen using modified EPA 1694 method for water sample extraction

Sample Name	Sample Mean Concentration (ppm)	Coefficient of Variation	Spiked Concentration (ppm)	Average Recovery Rate
Dihydrotestosterone (DHT)	3.49	46.27%	5.80	60.05%
17β-estradiol (E2)	4.45	5.19%	5.45	81.71%

Table 2.4. Estrogenic and Androgenic Equivalents of Fill & Draw Bioreactors Effluent

	Experiment 1 (TAS biomass)						Experiment 2 (MBR biomass)					
	EEQ (ng/L)			AEQ (ng/L)			EEQ (ng/L)			AEQ (ng/L)		
Time	AS	AS+	-Ctrl	AS	AS+	-Ctrl	AS	AS+	-Ctrl	AS	AS+	-Ctrl
0 h	nd	332.66 (±182.12)	0.11 (±0.09)	nd	896.17 (±325.60)	53.93 (±25.25)	0.01 (±0.01)	5433.70 (±372.8)	29.83 (±1.52)	nd	500.57 (±138.46)	320.86 (±46.01)
24 h	nd	1.64 (±0.88)	0.02 (±0.01)	nd	nd	36.00 (±5.03)	0.07 (±0.11)	1.06 (±0.82)	17.79 (±0.76)	nd	nd	195.44 (±8.73)
48 h	-	-	-	-	-	-	nd	nd	18.98 (±0.65)	nd	nd	146.49 (±29.80)
72 h	nd	nd	0.04 (±0.02)	nd	nd	22.04 (±11.70)	nd	nd	13.36 (±8.11)	nd	nd	207.96 (±59.75)
168 h	nd	nd	nd	nd	nd	nd	nd	0.32±0.51	8.56 (±1.33)	nd	nd	345.19 (±117.00)

Mean ± SD.
nd: non-detectable.

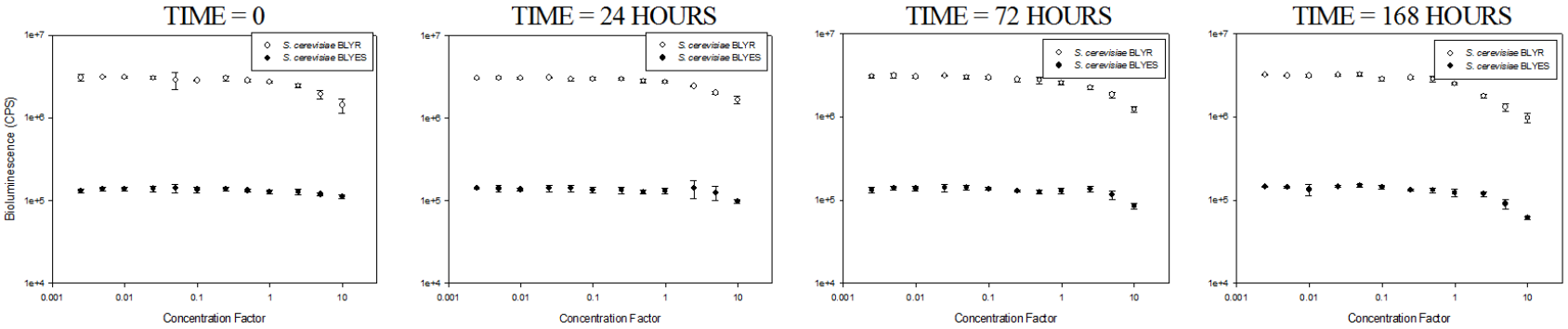
Fig.2.4 Time course analysis of bioluminescent yeast endocrine disruptor screening of lab-scale fill & draw wastewater treatment effluents. Experiment 1 used the activated sludge biomass from TAS, experiment 2 used the biomass from MBR (see also Table 2.1). In each plot, Y axis represents logged bioluminescence readings, X axis represents logged sample concentrations (from low to high). The point curve on the upper side was obtained from *Saccharomyces cerevisiae* BLYR for monitoring sample toxicity. Reduced bioluminescence in BLYR assay at higher concentration indicates the presence of cytotoxicity. The point curve on the lower side was obtained from *Saccharomyces cerevisiae* BLYES or BLYAS strain for detecting the presence of estrogenic or androgenic compounds, respectively.

Time course analysis of bioluminescent yeast endocrine disruptor screening of lab-scale Fill & Draw wastewater treatment effluents

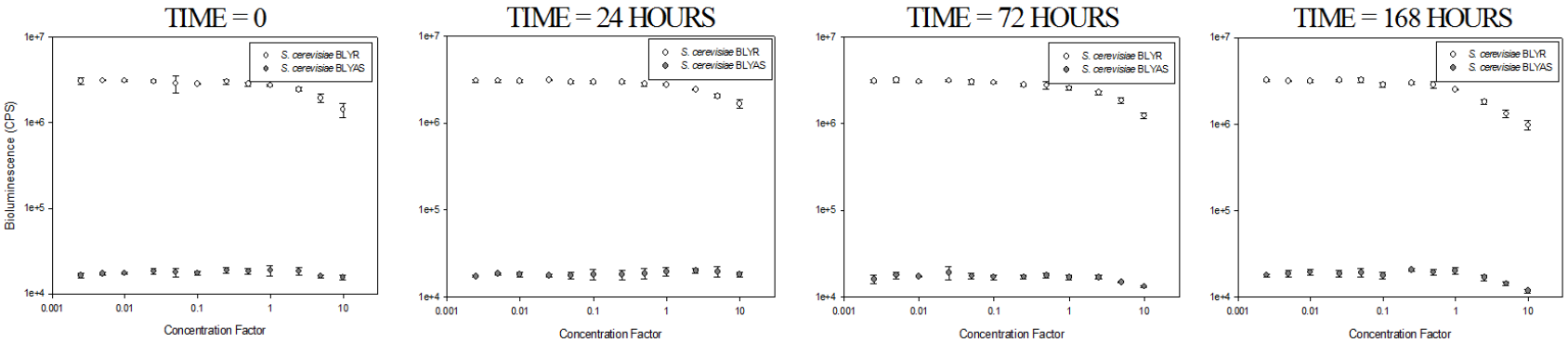
1a

Unspiked Bioreactor Samples (Experiment 1)

Estrogen Bioreporter



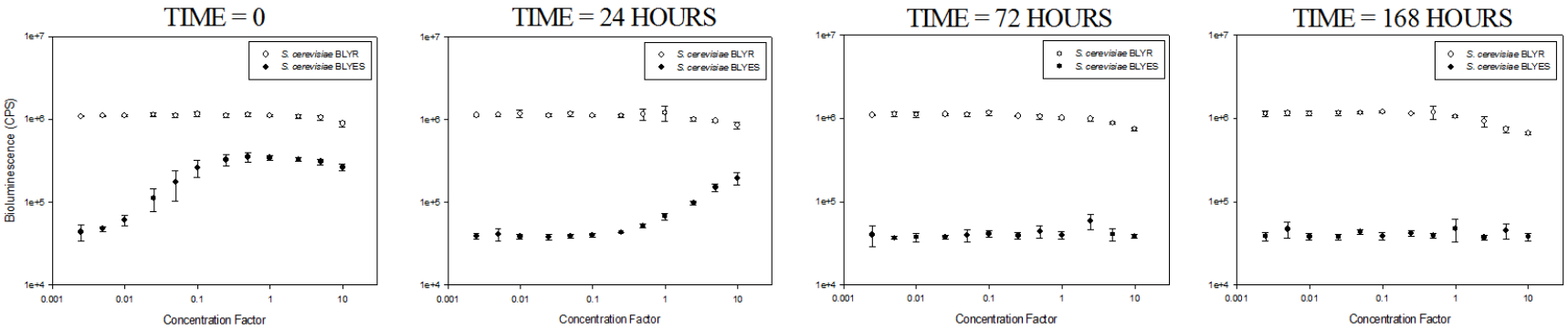
Androgen Bioreporter



1b

Spiked Bioreactor Samples (Experiment 1)

Estrogen Bioreporter



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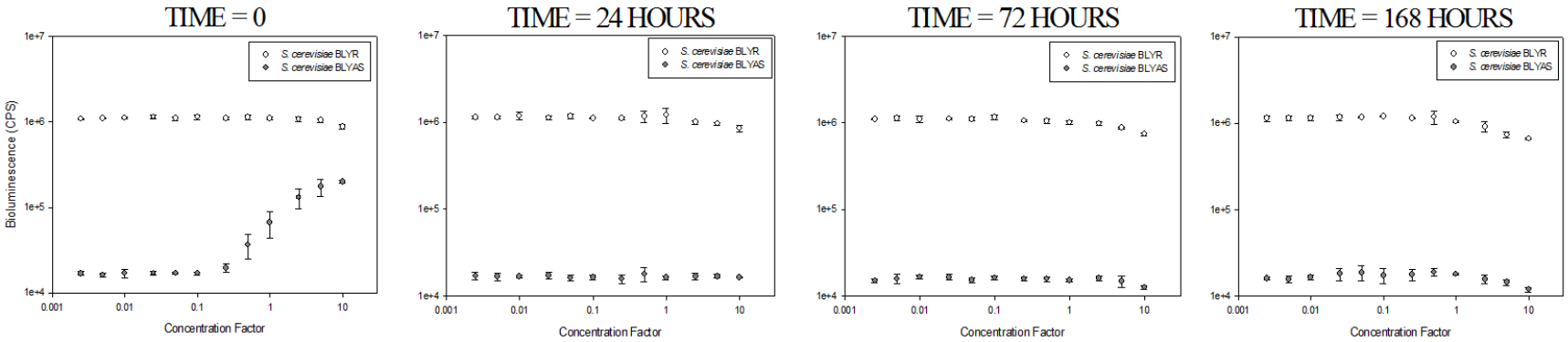
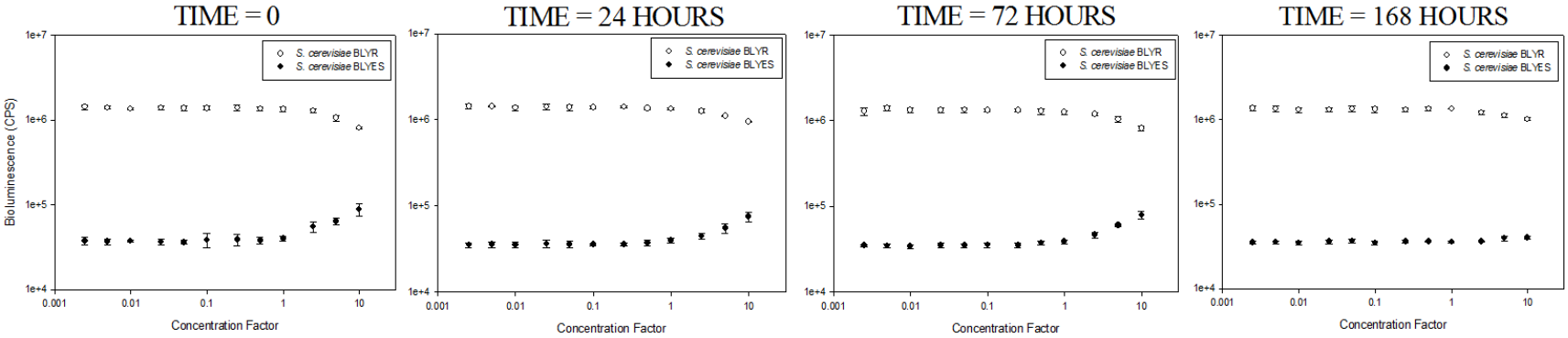


1c

Filtered Raw Wastewater Samples (Experiment 1)

Estrogen Bioreporter



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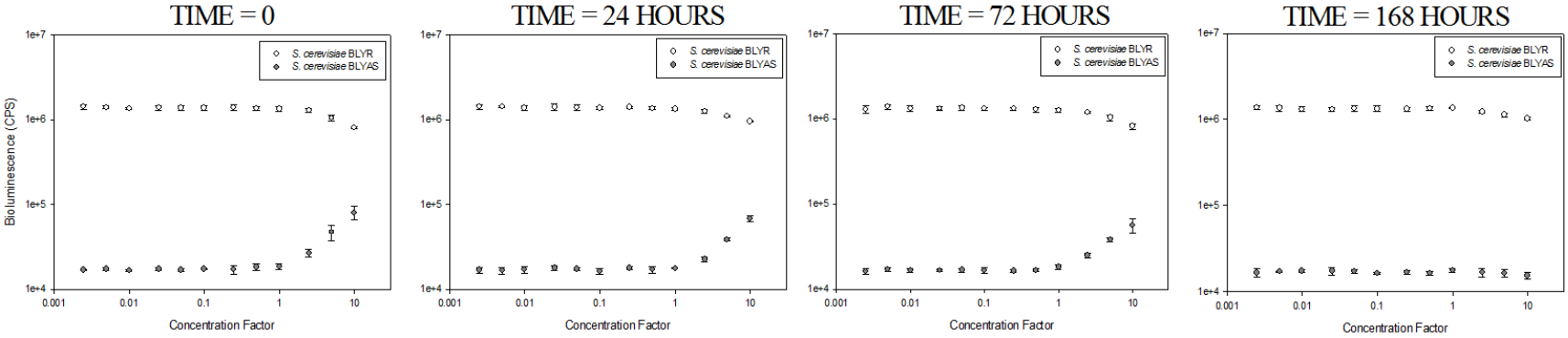
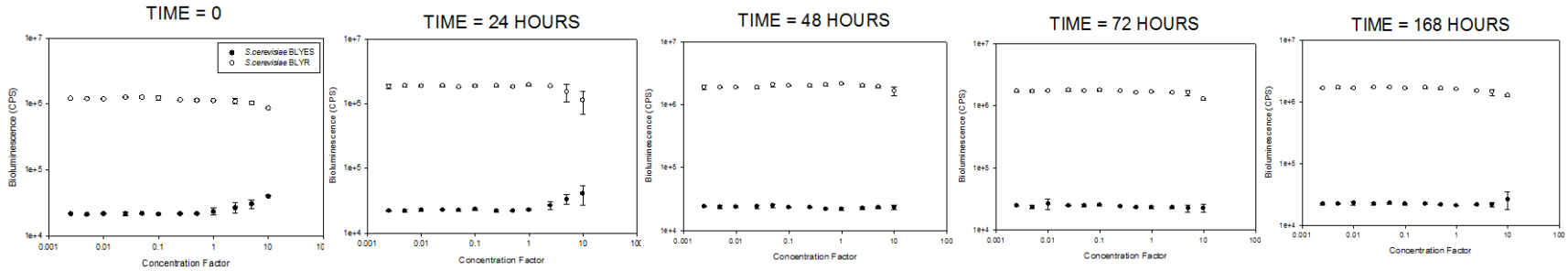


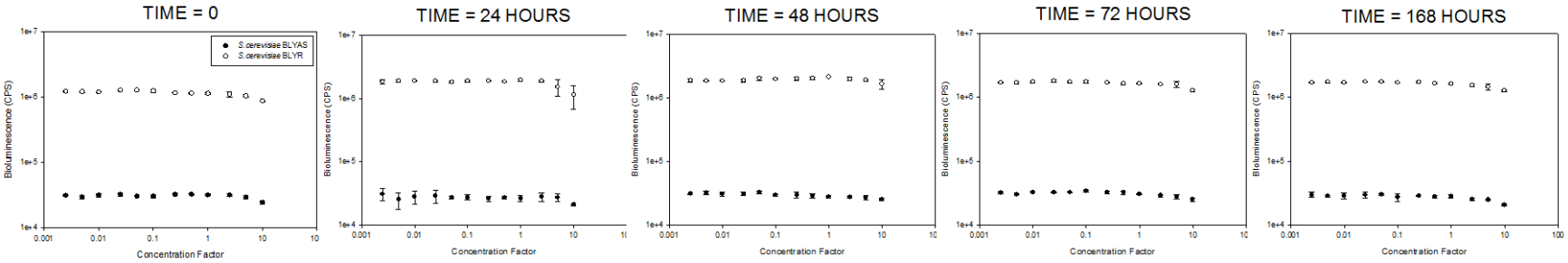
2a

Unspiked Bioreactor Samples (Experiment 2)

Estrogen Bioreporter



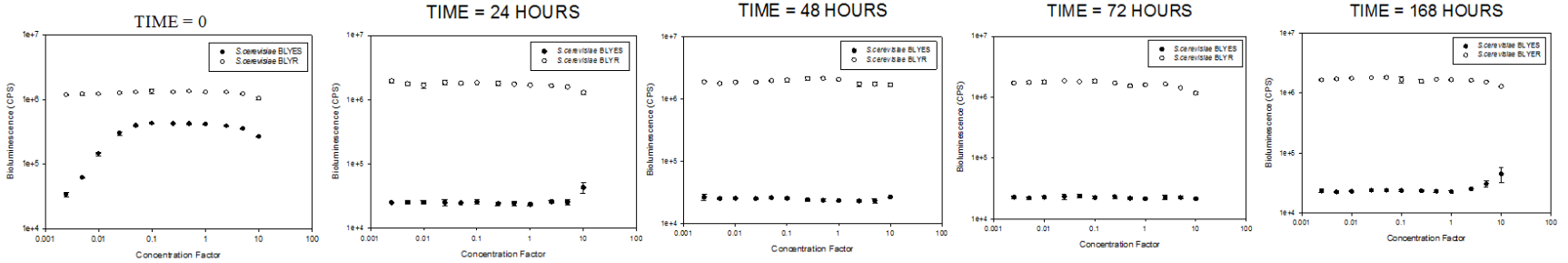
Androgen Bioreporter



2b

Spiked Bioreactor Samples (Experiment 2)

Estrogen Bioreporter



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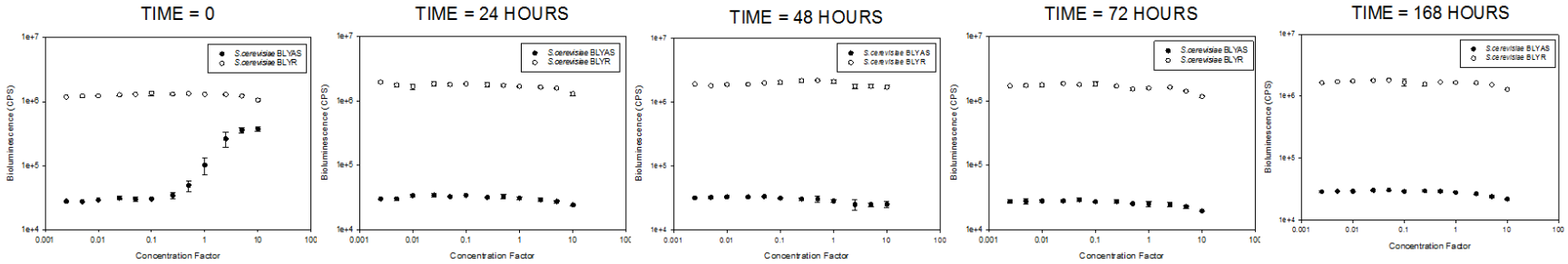

2c

Filtered Raw Wastewater Samples (Experiment 2)

Estrogen Bioreporter

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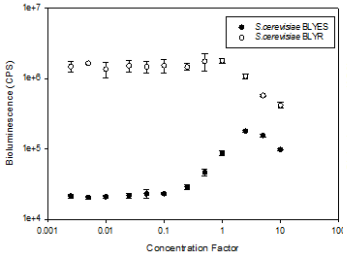

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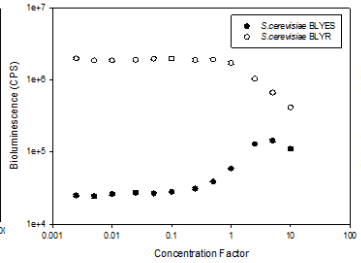
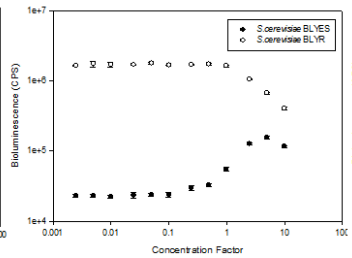
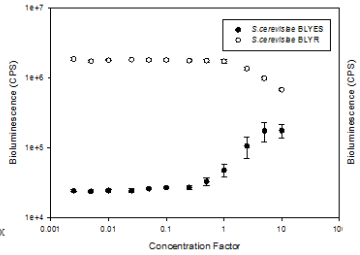


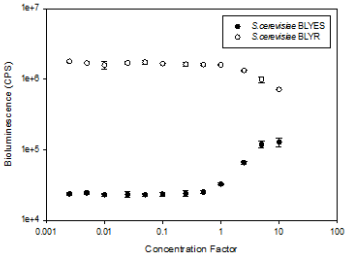
TIME = 48 HOURS



TIME = 72 HOURS



TIME = 168 HOURS



Androgen Bioreporter

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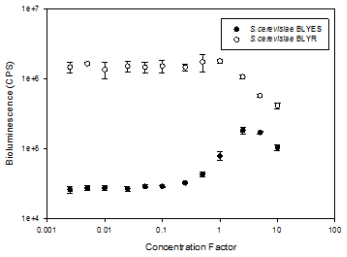
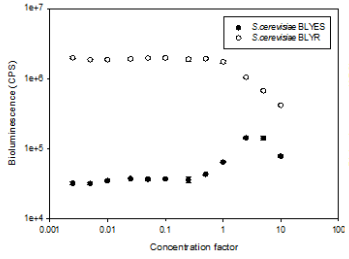


TIME = 24 HOURS



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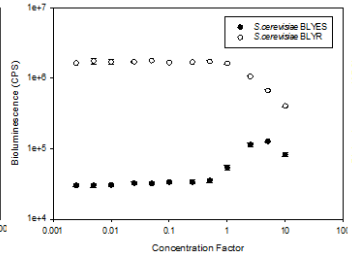
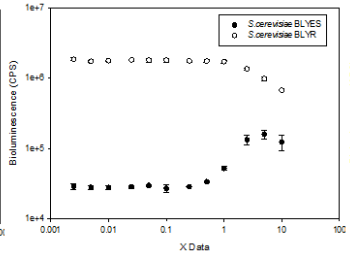
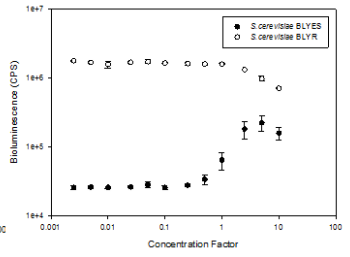


TIME = 72 HOURS



TIME = 168 HOURS



and 168 hours, no estrogenic responses were detectable. Considering the rapid removal of estrogenic response in the first experiments and the higher MLSS in MBR as compared to TAS (8500 mg/L vs. 2500 mg/L), in the 2nd fill & draw experiments, 10 times higher concentration of E2 (1 mM) was spiked and an additional sampling time at 48 h was added. The estrogenic activity of the filtered raw wastewater in the second experiment was much higher than the first experiment; the EEQ of the negative control at time 0 averaged 29.83 ng/L in the second experiment compared to 0.11 ng/L in the first experiment. As a result, the EEQ at time 0 in the spiked MBR bioreactors was significantly higher than that of the spiked TAS bioreactors at this time point (5433.70±372.8 ng/L vs. 332.66±182.12 ng/L, Table 2.4). Despite higher input of hormonal compounds, MBR biomass demonstrated very high efficiency in removing estrogenic activities. At 24 hour, the EEQ in spiked MBR bioreactors decreased to 1.06±0.82 ng/L. At 48 and 72 hours, no response was detectable in spiked MBR bioreactor effluent. However, at 168 hours, light response was detected, which could be the result of reduced biomass concentrations, as MLSS in the spiked MBR reactors decreased from 8666 mg/L to 666 mg/L after 7 days of fill & draw operations (Fig. 2.5).

Androgenic activities were detected in the negative controls (filtered raw wastewater) in both experimental groups. In the first experiments, initial AEQ averaged 0.11 ng/L while in the second experiments, initial AEQ averaged much higher at 320.86 ng/L. Both TAS and MBR biomass successfully removed the androgenic compounds in the filtered raw wastewater to below detectable level, as no androgenic activities were detected in the unspiked bioreactors in both groups of experiments throughout all the time points. Moreover, although strong androgenic response was detected at time 0 in both spiked TAS and MBR bioreactors due to spiked DHT, no

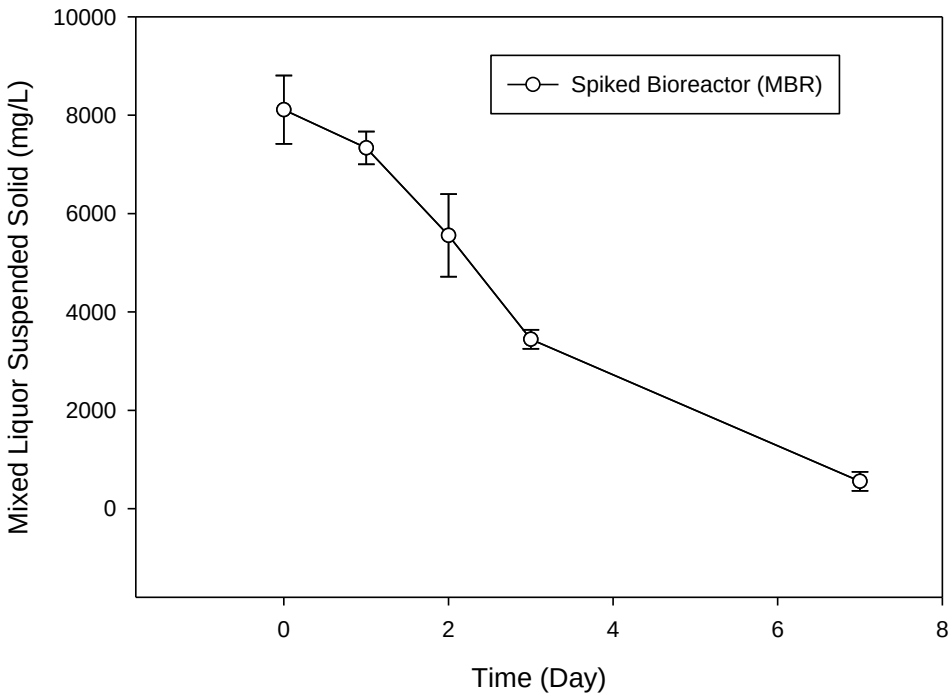


Fig 2.5. Mixed liquor suspended solid (MLSS) in spiked MBR bioreactors at 0, 1, 2, 3 and 7 day.

androgenic activates were detected after 24 hours in both experiments. Despite of 10 fold higher level of spiked DHT into the MBR bioreactors, the AEQ at time 0 (500.57±138.46 ng/L) wasn't higher than that of the spiked TAS bioreactor (896.17±325.60 ng/L) at this time point, suggesting a strong initial adsorption to the solid phase occurred. As the sampled MBR activated sludge had over 3 times higher TSS concentration than that of the TAS, this may explain why the initial AEQ in the spiked MBR reactors was not higher than that of the TAS reactors.

At the beginning of both experimental conditions, high concentration of estrogen and androgen were spiked into the bioreactors. Although the samples at time 0 were obtained immediately after the spike, the EEQ and AEQ in the spiked reactors at time 0 were significantly less than the spiked concentration, suggesting a rapid removal of hormones from the water phase occurred. This rapid removal was likely the result of adsorption to the biosolid, as two previous studies using activated sludge lab bioreactor also showed that the majority of spiked E2 and EE2 were instantly removed from water phase to solid phase by adsorption (Hashimoto and Murakami 2009; Suzuki and Maruyama 2006). During the 2nd fill & draw experiments, MLSS in the spiked bioreactors were measured (Fig. 2.5). The MLSS at the beginning of the experiment averaged 8666.6 mg/L, and continuously decreased along with the fill & draw operation each day, reaching an average of 666.6 mg/L by the end of the experiment. Despite the significant decrease of MLSS, the ability of the activated sludge in removing estrogenic and androgenic compounds was scarcely affected. Considering the high level of hormones added, it is likely that the microbial community adapted to the degradation of spiked hormones.

In the fill & draw bioreactor experiments, using filter sterilized raw wastewater as the refill allowed the activated sludge biomass to receive the same influent as in the field, which contains

complex organic contents including hormonal active compounds. Spiking E2, EE2 and DHT along with the filtered raw wastewater simulated the sudden increase of estrogenic and androgenic compounds in the wastewater and challenged the yeast bioluminescence reporters' ability to detect the change of hormonal activities in the samples. Overall, yeast reporters successfully detected the decrease of total endocrine disruptive activity in the treated wastewater during the fill & draw experiment. Coupled with the modified EPA 1694 sample prep method, yeast bioreporters produced monitoring results with good reproducibility (CV ranged from 3% to 172%) in 4 hours of sample exposure, proving their suitability to rapidly and effectively gauge the level of endocrine activities in wastewater samples.

Total androgenic, estrogenic activities and cytotoxicity in MBR and TAS wastewater effluents at HPUD

MBR and TAS effluent samples from week 1 to 27 were tested by BLYAS for androgenic responses. However BLYAS assay didn't detect any significant androgenic response in all effluent samples, suggesting that androgenic compounds in both MBR and TAS effluents were consistently at below detection level. Therefore BLYAS assay was excluded for the effluent samples after the 27^{th} week. The undetectable level of androgenic compounds in both MBR and TAS effluent was also in agreement with our findings in the fill & draw experiments, in which high level of spiked DHT in both MBR and TAS activated sludge were quickly removed within 24 hours and the level of androgenic compounds remained below detection level throughout the 7 days experiment period.

Estrogenic equivalents data for both TAS and MBR effluent field sample are presented in Fig. 2.6. All MBR and TAS effluent samples from June, 2011 to June 2012 was evaluated by BLYR and BLYES assay respectively for cytotoxicity and estrogenic activities The calculated estrogenic equivalents, lowest detectable concentration (LDC) and IC_{20} are listed in Table 2.2. In our study, all wastewater samples demonstrated various level of cytotoxicity. The IC_{20} of MBR effluent ranged from 1.15 to 194.94, and averaged 64.57. In comparison, the IC_{20} of TAS effluent ranged from 2.05 to 85.31 with an average of 36.05, indicating higher overall toxicity than MBR effluent. The IC_{20} of MBR and TAS had moderate correlation of 0.58 (Table 2.5), suggesting the variation of influent toxicity was one of the key contributing factors for effluent toxicity level. When comparing MBR with TAS in each individual day, the toxicity of MBR effluent was lower than that of TAS effluent on 22 of the 31 sampling dates, suggesting that MBR in general can achieve better cytotoxicity removal.

All TAS effluent samples showed estrogenic responses in BLYES assay throughout the 12 months of monitoring (Fig. 2.6, Table 2.2). The calculated EEQ_{50} for TAS effluent ranged from 21.61 ng/L to 0.04 pg/L, with an average of 3.25 ng/L and 4.33 ng/L SD. Most MBR effluent samples showed detectable estrogenic responses in BLYES assay except 3 of the 31 sampling dates. Compared to TAS, the calculated EEQ_{50} for MBR effluent ranged from 2.88 ng/L to 0.0134 pg/L, with approximately an order of magnitude lower average of 0.32 ng/L and 0.64 ng/L SD. The level of effluent EEQ in our study agrees with the results in several previous investigations conducted in different countries(Holbrook et al. 2002; Kirk et al. 2002; Murk et al. 2002; Svenson et al. 2003; Tanaka et al. 2001; Vethaak et al. 2005). In these studies, bioassays including YES, E-screen and ER-CALUX were used and the estimated WWTP effluents EEQ varied from <0.1 to 29.8 ng/L.

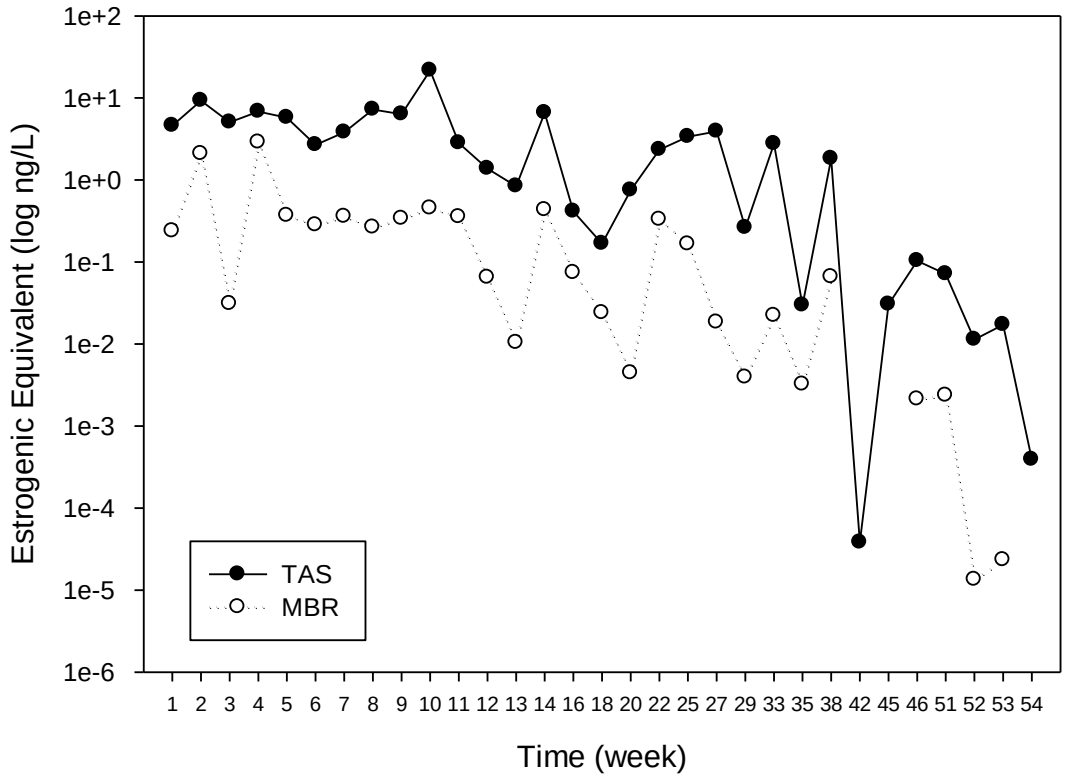


Fig 2.6. Estrogenic equivalent (EEQ_{50}) of TAS and MBR effluent from all field sampling dates. (see also Table 2.2).

Table 2.5. Pearson's correlation coefficient between effluent EEQ and key WWTP operational parameters.

	EEQ50 TAS (ng/L)	TAS EEQ50 removal	EEQ50 MBR (ng/L)	MBR EEQ50 removal	EEQ20 TAS (ng/L)	TAS EEQ20 removal	EEQ20 MBR (ng/L)	MBR EEQ20 removal	LDC TAS	LDC MBR	IC20 TAS	IC20 MBR
EEQ50 TAS (ng/L)												
TAS EEQ50 removal	**-1.00****											
EEQ50 MBR (ng/L)	0.42*	-0.42*										
MBR EEQ50 removal	-0.44*	0.44*	**-1.00****									
EEQ20 TAS (ng/L)	**0.99****	**-0.99****	0.39*	-0.42*								
TAS EEQ20 removal	**-0.99****	**0.99****	-0.39*	0.42*	**-1.00****							
EEQ20 MBR (ng/L)	0.43*	-0.43*	**0.98****	**-0.98****	0.41*	-0.41*						
MBR EEQ20 removal	**-0.46****	**0.46****	**-0.98****	**0.98****	-0.43*	0.43*	**-1.00****					
LDC TAS	-0.38*	0.38*	-0.38*	0.25	-0.40*	0.40*	-0.39*	0.26*				
LDC MBR	-0.35	0.35	-0.30	0.30	-0.33	0.33	-0.34	0.34	0.36			
IC20 TAS	0.07	-0.07	0.06	-0.11	0.09	-0.09	0.12	-0.18	-0.44*	0.21		
IC20 MBR	0.20	-0.20	-0.09	0.02	0.20	-0.20	-0.03	-0.04	-0.40*	-0.07	**0.58****	
TAS MLSS (mg/L)	-0.09	0.09	-0.27	0.27	-0.03	0.03	-0.25	0.26	0.10	0.15	-0.04	0.07
TAS MLVSS (mg/L)	-0.13	0.13	-0.34	0.34	-0.07	0.07	-0.33	0.33	0.10	0.18	-0.07	0.10
TAS SRT (day)	-0.03	0.03	-0.25	0.25	0.02	-0.02	-0.26	0.25	0.06	-0.05	0.06	0.11
TAS HRT (hour)	0.24	-0.24	-0.01	0.02	0.20	-0.20	0.05	-0.03	0.28	-0.25	-0.38*	-0.18
TAS F/M Ratio	-0.04	0.04	0.28	-0.29	-0.07	0.07	0.26	-0.26	-0.15	0.11	0.18	-0.04
MBR MLSS (mg/L)	-0.30	0.30	-0.22	0.22	-0.31	0.31	-0.20	0.21	0.10	0.08	-0.08	-0.01
MBR MLVSS (mg/L)	-0.31	0.31	-0.25	0.25	-0.33	0.33	-0.24	0.24	0.10	0.13	-0.07	0.04
MBR SRT (day)	-0.02	0.02	-0.07	0.06	0.02	-0.02	-0.13	0.12	-0.16	-0.11	0.16	0.31
MBR HRT (hr)	0.07	-0.07	0.25	-0.22	0.02	-0.02	0.22	-0.19	0.17	-0.16	-0.27	-0.09
MBR F/M Ratio	0.10	-0.10	-0.14	0.14	0.08	-0.08	-0.08	0.08	0.05	-0.05	-0.08	-0.04
RAIN (inches per 24hr)	-0.24	0.24	-0.15	0.15	-0.20	0.20	-0.18	0.18	-0.04	**0.77****	0.20	0.00
TREATED PLANT INFLUENT (MGD)	-0.16	0.16	-0.11	0.09	-0.11	0.11	-0.15	0.12	-0.19	0.31	0.38*	0.22
TOTAL PLANT EFFLUENT (MGD)	-0.14	0.14	-0.11	0.09	-0.09	0.09	-0.15	0.12	-0.20	0.35	0.36*	0.21
EFFLUENT TEMP. (CELSIUS)	0.34	-0.34	0.27	-0.26	0.27	-0.27	0.31	-0.30	0.13	**-0.47****	-0.37*	-0.28

Table 2.5. Continued

	EEQ50 TAS (ng/L)	TAS EEQ50 removal	EEQ50 MBR (ng/L)	MBR EEQ50 removal	EEQ20 TAS (ng/L)	TAS EEQ20 removal	EEQ20 MBR (ng/L)	MBR EEQ20 removal	LDC TAS	LDC MBR	IC20 TAS	IC20 MBR
cBOD5 Plant Influent (mg/L)	0.26	-0.26	0.34	-0.33	0.21	-0.21	0.39*	-0.38*	0.08	-0.21	-0.22	-0.16
cBOD5 Plant Effluent (mg/L)	0.04	-0.04	-0.31	0.23	0.04	-0.04	-0.28	0.20	-0.19	0.38*	0.38*	0.36*
Suspended Solid Plant Influent (mg/L)	0.15	-0.15	0.40*	-0.40*	0.09	-0.09	0.43*	-0.43*	0.00	-0.22	-0.15	-0.21
Suspended Solid Plant Effluent (mg/L)	-0.20	0.20	-0.15	0.13	-0.18	0.18	-0.17	0.14	-0.06	0.39*	0.31	0.09
Settled Solid Plant Influent (mL/L)	0.19	-0.19	0.43*	-0.43*	0.13	-0.13	0.46*	**-0.46****	-0.03	-0.24	-0.14	-0.18
Dissolved Oxygen Plant Influent (mg/L)	**-0.48****	**0.48****	-0.39*	0.38*	-0.44*	0.44*	-0.43*	0.41**	0.06	**0.57****	0.22	0.22
Dissolved Oxygen Plant Effluent (mg/L)	-0.27	0.27	-0.37	0.37	-0.23	0.23	-0.40*	0.40*	0.01	**0.59****	0.29	0.09
Ammonia Plant Influent (mg/L)	0.12	-0.12	0.05	-0.03	0.10	-0.10	0.07	-0.04	0.27	-0.08	**-0.49****	-0.23
Ammonia Plant Effluent (mg/L)	**0.79****	**-0.79****	0.04	-0.05	**0.80****	**-0.80****	0.03	-0.04	-0.12	-0.12	-0.13	-0.07

* Significant at $p < 0.05$; ** Significant at $p < 0.01$;

When comparing the EEQ_{50} of MBR and TAS effluent on each sampling date (Fig. 2.6), the MBR at HPUD consistently produced effluent with less estrogenic activity than the TAS throughout the monitoring period. The average estrogenic equivalent from MBR effluent was about 10 times less than that of TAS. Several previous studies featuring either lab-scale or full-scale MBR and TAS on different locations also suggested that MBR can deliver enhanced removal performance for several different type of EDCs (Clara et al. 2005b; Gaulke et al. 2009; Radjenović et al. 2009; Vega-Morales et al. 2013). The findings in our study add further evidence that MBR is more effective in EDCs removal, and the conclusion is based on the long-term 12 months monitoring on full-scale TAS and MBR operating in parallel with the same source of influent.

In order to evaluate the variation of EDCs level in wastewater effluent, in the last three sampling dates (week 52 to 54), triplicate sampling, extraction and assay were performed on the MBR and TAS effluents. The coefficient of variation (CV) for TAS EEQ_{50} was calculated as 17%, 78% and 138% for week 52, 53 and 54 respectively. While the CV for MBR EEQ_{50} were 89% and 81% for the detectable week 52 and 53 respectively. These results demonstrated that our wastewater extraction and yeast assay protocol has relatively small variation when testing field wastewater sample, and is able to gauge and differentiate hormonal activities changes at least on the magnitude level.

EEQ is a well-recognized metric to describe the level of estrogenic activity for a certain chemical or sample. Generally EEQ is obtained by dividing the EC_{50} of 17β-estradiol with the EC_{50} of the sample. However when calculating EEQ for environmental samples, special care should be taken as cytotoxicity in the samples could greatly influence the end result. In our study, although wastewater samples were concentrated 1000 times, most samples showed significant response

in BLYES assay without producing a full sigmoidal curve. Therefore in sample EC_{50} calculation, we used the max and min bioluminescence of the 17β-estradiol response curve instead of the sample to identify the half-maximal response value. Then the fitted linear equation of the responsive sample data points was used to extrapolate the EC_{50}. A potential drawback of this calculation is that when the sample contains high cytotoxicity, bioluminescence response at high concentration is inhibited, which yields smaller slope in the fitted linear equation, causing overestimated EC50 and underestimated EEQ. This bias becomes more prominent when sample has weaker estrogenicity. To address this problem, an alternative way of EEQ calculation is to use the EC_{20} of the sample and 17β-estradiol instead of the EC_{50}. In theory, reduced slope has less impact on EC_{20} than EC_{50} (see Fig. 2.3). Therefore EEQ calculation based on EC_{20} could help alleviate the bias caused by sample toxicity. To compare these two calculations, we calculated the EEQ for all the effluent samples using both EC_{50} and EC_{20}, and named them as EEQ_{50} and EEQ_{20} respectively. EEQ_{20} showed nearly identical trend as EEQ_{50}. The correlation between the EEQ_{50} and EEQ_{20} for TAS and MBR was 0.99 and 0.98 respectively (Table 2.5). As expected, the main differences between EEQ_{50} and EEQ_{20} lie in samples with low estrogenic activities (Table 2.2 and Fig. 2.7). For both MBR and TAS samples, EC_{20} based calculation significantly elevated the EEQ values for weak estrogenic samples. The minimum, lower quartile and media values of TAS EEQ all increased when calculated by EC_{20} (Table 2.6). Stronger trend was seen in the EEQ of MBR effluents as they contained less estrogenic activities. The distribution of MBR samples showed increased values between 0.5 and 1 in EEQ_{20}. The minimum EEQ of TAS effluent increased by 2989% from 3.82×10^{-5} to 1.18×10^{-3} ng/L while the minimum EEQ of MBR increased by 2810%

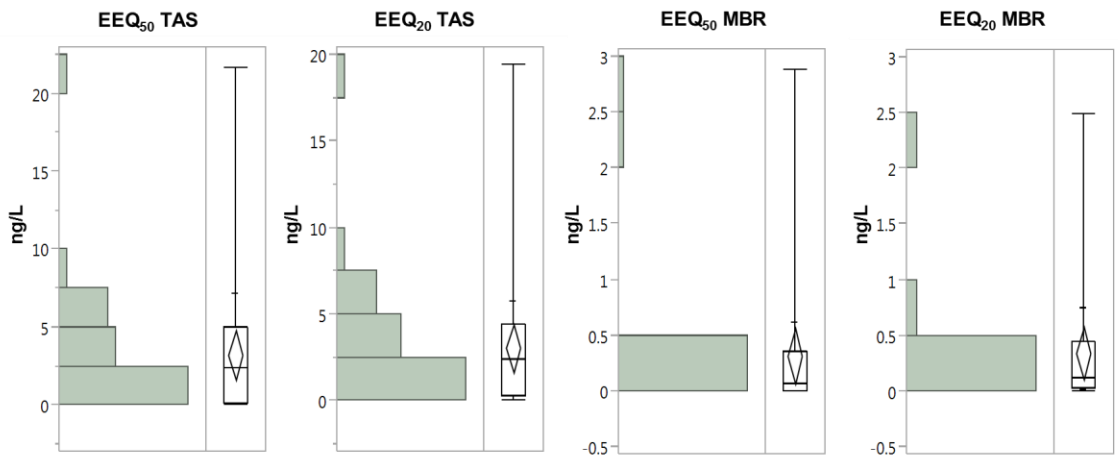


Fig 2.7. Distribution and boxplot for EEQ_{50} and EEQ_{20} of MBR and TAS effluent

Table 2.6. Descriptive statistics for EEQ_{50} and EEQ_{20} of MBR and TAS field effluent samples

	EEQ_{50} TAS (ng/L)	**EEQ_{20} TAS (ng/L)**	**EEQ_{50} MBR (ng/L)**	**EEQ_{20} TAS (ng/L)**
Maximum	21.61	19.38	2.88	2.49
75% percentile	5.03	4.43	0.35	0.45
median	2.33	2.44	0.07	0.12
25% percentile	0.10	0.30	0.01	0.03
minimum	3.82E-05	1.18E-03	1.34E-05	3.90E-04
Mean	3.25	3.07	0.32	0.35
Std. Dev	4.33	3.80	0.64	0.61

from 1.34×10^{-5} to 3.90×10^{-4} ng/L. These findings suggest that EC20 based EEQ may to be more suitable for describing the estrogenic activity in toxic environmental samples.

It's worth noting that EEQ calculation for wastewater samples was based on the assumption that there were no cytotoxicity or inhibitory compounds present, and the sample can be further concentrated to allow the estrogenic response of the *in vitro* assay reach the same maximum level as 17β-estradiol. In actual wastewater samples, these assumptions are unlikely to be true, since toxic, antagonistic or synergistic effects could all be present. Therefore when interpreting EEQ/AEQ from *in vitro* assays, it is necessary to understand that the measurement is reflecting a combined effect. The link between bioassay and chemical analysis in WWTP EDCs detection were discussed by many researchers. While some studies found natural estrogens E1, E2, E3 and synthetic estrogen EE2 detected in chemical analysis can account to up to 98% of estrogenic activities determined in yeast or mammalian cell based bioassays, some also found over or under representation of estrogenicity by compounds identified in targeted-detection(Liu et al. 2009). A previous study also found that androgenic activity detected by yeast based androgen assay could not be fully explained by the compounds detected in chemical analysis(Drewes et al. 2006). These studies suggest that chemical analysis alone isn't sufficient to comprehensively evaluate the hormonal activity in wastewater samples. A previous study conducted receiver operating characteristic analysis to assess the sensitivity and specificity of *in vitro* assays including YES, human reporter gene assay and e-screen for estrogenic compound detection and concluded that all evaluated *in vitro* assays were able to effectively predict the *in vivo* effects of tested compounds (Martin et al. 2005). Since the contents of wastewater could differ considerably across time and geological locations, and the fate of different EDCs can be influenced by many

factors in wastewater treatment, *in vitro* bioassay should be incorporated as a routine analysis for WWTP performance evaluation.

Relationship between Operational Parameters and residual EDCs in effluent

The operational parameters of HPUD wastewater facility on the sampling dates along with the tested EEQ, IC_{20} are summarized in Supplemental Table 2.1. Parameters that were monitored separately for TAS and MBR included MLSS, MLVSS, SRT, HRT and F/M ratio. Other operational parameters monitored for the influent and effluent of the entire plant included the total flow, $cBOD_5$, suspended and settled solid, dissolved oxygen and ammonia content. To determine whether these operational parameters are responsible for the variation of EEQ level in the effluent, Pearson's correlation analysis was performed (Table 2.5). MBR and TAS EEQ removal efficiencies were also included in the correlation analysis. To obtain EEQ removal efficiency, we assumed a steady state influent EEQ concentration equivalent to 29.82 ng/L, which was measured on the raw wastewater used the 2nd fill & draw experiments (Supplemental Table 2.1). No significant correlation was found between effluent EEQ, EEQ removal efficiency and key operational parameters MLSS, MLVSS, SRT and HRT for either MBR or TAS. Similar findings on the influence of WWTP operational parameters on EDC removal was discussed in several previous studies as well. SRT is one of the critical parameters for the bioremoval activity of activated sludge. One study suggested that the SRT above 10 days can increase the quantity of nitrification bacteria and provide higher removal of micropullutants including natural estrogens and bisphenol A(Clara et al. 2005a). While another study found that SRT above 2 days yielded similar high EDCs removal, and no significant improvement was found with longer SRT(Drewes et al. 2006). A third study also claimed no statistically significant correlation between EDCs removal and SRT, though they

did find that WWTPs with either high SRT or nitrification activity were more effective on hormone removal (Servos et al. 2005). In our study, SRT for TAS and MBR were all above 2 days, and the majority of dates had high SRT over 10 days. SRT variation above these critical values were found with minimal impact on the effluent EDCs level. This may explain why no correlations were found between the estimated effluent EDCs level/removal efficiency and SRT.

HRT is the amount of time wastewater is exposed to the biosolid for biodegradation and adsorption. In our study, no substantial correlation was found between HRT and effluent EEQ level/removal efficiency. Previous studies also found no statistically significant correlation between HRT and EDCs removal efficiency, though they do find that higher HRT can exert positive effect on EDCs removal (Drewes et al. 2006; Servos et al. 2005).

A previous study reported good correlation between BOD removal and EDCs removal in secondary treatment(Drewes et al. 2006). In our study, BOD was measured on the combined effluent rather than separately for MBR and TAS, and we found no correlation between both estimated EEQ and removal efficiency of MBR/TAS and BOD removal. Although BOD removal was highly efficient at HPUD (removal rate ranged from 93.3% to 99.5%, averaging 98.3%), various level of EEQ was still present in the effluent, suggesting that effluent BOD level is not an accurate indicative index for EDCs level, and dedicated EDCs assay is necessary for more appropriate estimation.

As the MBR and TAS at HPUD process the same source of wastewater influent, they provided the unique opportunity for long-term direct field comparison between these two technologies. Typically MBR technology allows longer SRT and higher MLSS than TAS(Drewes et al. 2006). In our study, MBR operated at similar average SRT as TAS did, but maintained significantly higher

MLSS level in all sampling dates. The average MLSS in MBR averaged 5447.7 mg/L, which was more than two times higher than the average MLSS in TAS (2506.8 mg/L). Despite having lower average HRT compared to TAS (16.21 vs. 31.10 hrs), MBR effluent still consistently demonstrated less estrogenic activity and toxicity than TAS. This result is also in line with the findings in our fill & draw experiments, in which more concentrated MBR biosolid demonstrated faster hormone removal than TAS biosolid. Although previous studies also suggested that higher MLSS in MBR contributes to its improved EDCs removal(Drewes et al. 2006; Hu et al. 2007; Judd 2008), whether this is the sole contributing factor to the ~10 fold difference in effluent estrogenic activity at HPUD requires further investigation.

2.4 Conclusions

In this study, we demonstrated the use of standardized yeast bioluminescent assays as high-throughput screening tools to monitor the estrogenic, androgenic potencies and toxicity in the wastewater treatment systems. To evaluate the sensitivity and reproducibility of the yeast assay in wastewater monitoring, we conducted 7 days semi-continuous batch reactor experiments using the activated sludge and raw wastewater spiked with hormones. Yeast bioreporter assay successfully captured the rapid removal of estrogenic and androgenic activities in the bioreactors, and also demonstrated good reproducibility and sensitivity. This standardized assay was then applied in a 12 month comparative study on the wastewater effluent from the parallel-operated MBR and TAS located at Powell, TN, USA. The yeast assay detected no significant androgenic response in all tested TAS and MBR samples. While all TAS effluent samples and most (3 of the 31 dates) MBR effluent samples showed detectable estrogenic activity. The EEQ_{50} for TAS effluent

ranged from 21.61 ng/L to 0.04 pg/L and averaged 3.25 ng/L. For MBR, the effluent EEQ ranged from 2.88 ng/L to 0.0134 pg/L and averaged ~10 fold less (0.32 ng/L) than TAS. Despite the large temporal variation, the EEQ level in MBR effluent was consistently lower than that of TAS on any given sampling day. Most MBR effluent samples also contained less cytotoxicity than TAS. These data collectively suggest that MBR can significantly reduce the release of EDCs and toxic compounds into the environment. Further analysis revealed no significant correlation between effluent EEQ level and WWTP operational parameters including MLSS, SRT, HRT and BOD. Overall, our study demonstrated that the yeast bioluminescent assay is suitable to serve as a sensitive, high-throughput, rapid and low-cost EDCs screening tool for routine wastewater monitoring purpose.

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Chapter 3. Computational Modeling of Interactions between Human Estrogen Receptor Mutants and Endocrine Disruptors

Abstract

This chapter describes and reports the results from the computational modelling of the interaction between selected estrogenic EDCs and wildtype/mutant hERα ligand binding domain. Five point mutations (Asp351Tyr, Glu353Val, Met396Val, Leu525Ala and Asp538Gly) that are close or inside hERα ligand binding pocket were selected from previous literature and cancer database. Two forms of hERα ligand binding domain, the agonist form and antagonist form and their *in silico* mutated models were subjected to molecular dynamics (MD) simulation to explore potential conformation changes in the binding pocket as the result of point mutations . MD trajectories were clustered based conformation variation and one conformation was chosen from each of the cluster. Selected MD conformations were computationally docked with 29 selected environmental EDCs to evaluate the changes of predicted binding affinity as the result of point mutations.

3.1 Introduction

Human estrogen receptor alpha (hERα) features flexible binding pocket that many natural and artificial ligands besides natural estrogens can also bind and modulate the activation of the receptor and downstream transcriptional activity. Since these non-natural hormonally active compounds can directly impact and interfere the normal function of the endocrine system, they were commonly referred as endocrine disruptive chemicals (EDCs). As the public health concern of EDCs increased in the past decades, many research efforts were emphasized on the screening and estimation of EDC potency. These studies mostly focused on evaluating the response of the most common receptor form, the “wild type” human estrogen receptor, that is defined by

current understanding of the protein hERα. However in human genome, small genetic variations, often referred as single nucleotide polymorphism (SNPs) are found to be ubiquitously present. For hERα, studies have revealed numerous possible mutations in both the exons and introns of its gene ESR1 in both healthy and cancer patients, primary and metastatic tumors(Roodi, Bailey et al. 1995, Segal and Dowsett 2014, Thomas and Gustafsson 2015). Because mutations found in ER originated from a very limited population, and the incidence of mutations are far from uncommon, the past EDCs evaluation studies focusing solely on "wildtype" ER can be incomplete and biased. Consequently this raises a serious issue as to whether a susceptible subpopulation of hER mutants exist that demonstrates altered susceptibility or responses to estrogenic and xenoestrogenic chemicals in the environment. Therefore this study specifically investigated five mutations of hERα inside or close to the ligand binding pocket for their effect to the interaction between a broad spectrum of estrogenic EDCs and the protein receptors. The key questions that were asked are: 1). Does a mutation occurring inside or outside the ligand binding pocket of hER cause differential response to estrogenic EDCs? 2). If a mutation on hER causes change of response to natural estrogen E2, will the direction and degree of deviation also apply to other estrogenic EDCs? To address these two questions, we chose to use both computational modelling and an experimental yeast bioluminescent estrogen assay to evaluate and then test the binding and transactivation activity of wildtype and mutant hERα in the presence of EDCs from a wide range of origins.

This chapter describes and reports the results from the computational modelling of the interaction between selected estrogenic EDCs and wildtype/mutant hERα ligand binding domain. As crystal structures for hERα ligand binding domain became available, the ligand-protein binding

interaction was revealed for several compounds ranging from natural estrogens such as 17β-estradiol to anti-estrogen pharmaceuticals such as 4OH-tamoxifen etc.(Andrew, Danielle et al. 1998, David, Yong et al. 1998). While crystal structures provided fundamental insights to the interactions of ER and specific ligands, the rigid crystal structure is unable to provide comprehensive representation of the dynamic and flexible nature of the protein receptor *in vivo.* For ligand binding, critical conformations may be different from the crystal structure. In this study, molecular dynamics simulation were employed to simulate the dynamics of hERα protein *in vivo*. The aim of this study was to employ MD simulation to explore potential conformation changes at the binding pocket when point mutation occurs and to evaluate how these mutations could alter the binding affinity to estrogenic EDCs. It is hypothesized that computational modelling through MD simulation and ensemble virtual docking can predict binding affinity changes caused by mutations on the estrogen receptor, and the predicted binding changes could guide the direction of *in vitro* bioassay validation. Two forms of hERα ligand binding domain, the agonist form and antagonist form and their *in silico* mutated models were included in the MD simulation. MD trajectories were clustered to select snapshots from each of the cluster, which were then computationally docked with selected estrogenic ligands to evaluate the predicted binding affinity.

3.2 Materials and Methods

Ligand preparation

The structure of 29 estrogenic compounds (Table 3.1) were obtained from the PubChem and National Center for Toxicological Research Estrogen Receptor Binding Database (NCTRER)(Tong

2008). Structures were prepared using the "Protonate 3D" module of MOE 2012 (Molecular Operating Environment, Chemical Computing Group, Montreal, Canada) to add hydrogens with pH condition of 7, and then energy-minimized to 0.05 kcal/mol/Å RMS (root mean square) energy gradient using the MMFF94s force field(Halgren 1999) provided in MOE.

Protein structure preparation and *in silico* mutagenesis

Two x-ray crystal structures (PDB entry: 2B1Z, 3ERT) were selected for the docking and molecular dynamics simulation study. 2B1Z represents the agonist binding form of hERα ligand binding domain, while 3ERT represents the antagonist binding form of the ligand binding domain. Structural preparation and *in silico* mutagenesis were conducted in MOE 2012. Firstly, the ligands in 2B1Z and 3ERT structures were removed and one monomer of each protein structure was retained. Then the protein structures were prepared by "homology modeling" module to fix disconnected backbone, and then processed in the "structural preparation" module to add missing atoms including hydrogen, and protonate according to pK_a estimates of each residue at pH 7. To select the sites for mutagenesis study, previous findings of hER mutations reported in COSMIC cancer mutation database(Forbes, Bhamra et al. 2008) and literature were collected and reviewed. Eventually five mutation sites inside and closed to the ligand binding pocket were selected based on their position (Table 3.2, Fig 3.1). The mutations Asp351Tyr, Glu353Val, Met396Val, Leu525Ala and Asp538Gly were performed *in silico* separately on 2B1Z and 3ERT, generating a total of 10 structures. Structures were minimized with tether restraints of 0.5 to RMS gradient of 0.1 kcal/mol/$Å^2$ using MMFF94x force field implemented in MOE.

Table 3.1 Structure and relative binding affinity (experimental) of tested estrogenic compounds

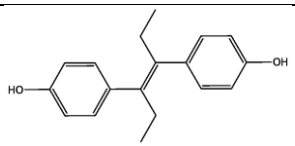
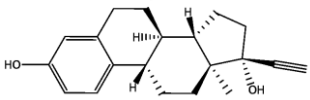
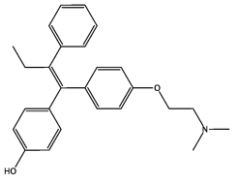
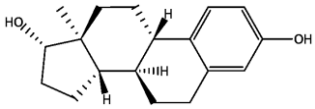
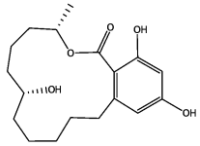
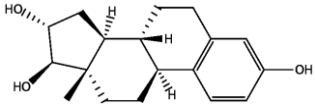
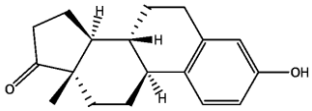
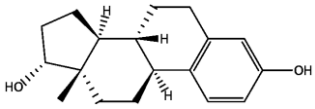
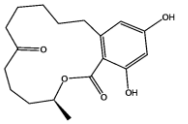
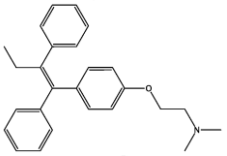
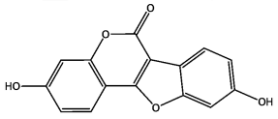
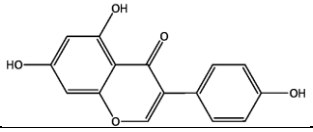
Compound	Structure	ER_RBA	Activity Score	Activity Category	Chemical Class
diethylstilbestrol (DES)		3.98E+02	100	active strong	Synthetic estrogen
ethynyl estradiol		1.91E+02	96	active strong	Synthetic estrogen
4-hydroxy-tamoxifen		1.74E+02	95	active strong	Anti-estrogen
17beta estradiol (E2)		1.00E+02	93	active strong	Natural estrogen
alpha zearalanol		3.02E+01	87	active strong	Phytoestrogen
estriol		9.77E+00	81	active strong	Natural estrogen
estrone		7.24E+00	80	active strong	Natural estrogen
17alpha estradiol		3.09E+00	76	active strong	Synthetic estrogen
zearalanone		2.09E+00	74	active strong	Phytoestrogen
tamoxifen		1.62E+00	73	active strong	Anti-estrogen
coumestrol		8.91E-01	70	active medium	Phytoestrogen
genistein		4.36E-01	66	active medium	Phytoestrogen

Table 3.1 Continued

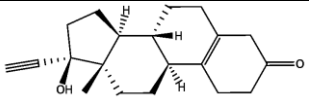
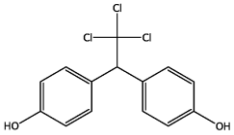
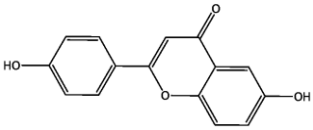
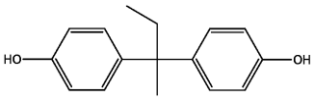
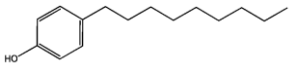
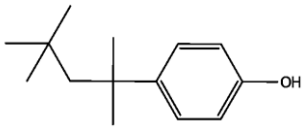
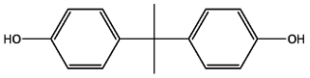
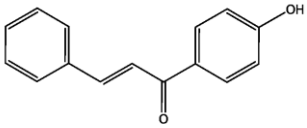
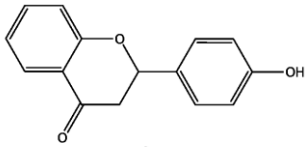
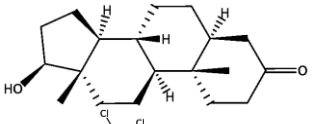
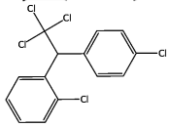
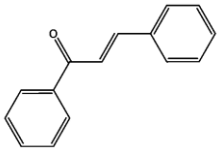
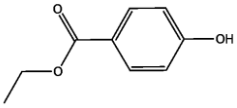
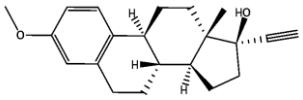
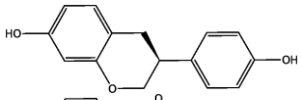
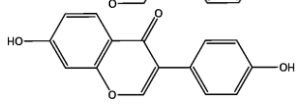
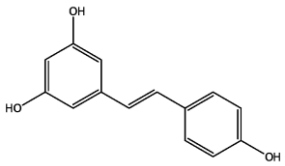
Compound	Structure	ER_RBA	Activity Score	Activity Category	Chemical Class
norethynodrel		2.14E-01	63	active medium	Synthetic estrogen
HPTE		2.51E-01	63	active medium	Pesticide metabolite
4',6-dihydroxyflavone		1.51E-01	61	active medium	Phytoestrogen
bisphenol B		8.51E-02	58	active medium	Plasticizer
nonylphenol		2.95E-02	53	active medium	Phenols Alkyl
4-t-octylphenol		1.50E-02	50	active medium	Phenols Alkyl
bisphenol A (BPA)		7.80E-03	46	active weak	Plasticizer
4'-hydroxychalcone		3.70E-03	43	active weak	Phytoestrogen
4'-hydroxy flavanone		2.23E-03	40	active weak	Phytoestrogen
5alpha dihydrotestosterone		1.30E-03	38	active weak	Androgen
o,p'-DDT		1.41E-03	38	active weak	Pesticide
chalcone		1.51E-03	38	active weak	Phytoestrogen
ethyl 4-hydroxybenzoate		6.00E-04	34	active weak	Phenols

Table 3.1 Continued

Compound	Structure	ER_RBA	Activity Score	Activity Category	Chemical Class
Mestranol		2.24E+00	74	active strong	Synthetic estrogen
***S*-equol**		1.51E-01	61	active medium	Phytoestrogen
daidzein		2.20E-02	52	active medium	Phytoestrogen
resveratrol		na	na	na	Phytoestrogen

na: no data available.

ER_RBA: estrogen receptor binding affinity (relative to 17β-estradiol) measured by rat uterine cytosol estrogen receptor competitive receptor binding assay. Natural estrogen E2 has ER_RBA = 100. ER_RBA > 100 means ligand's binding affinity is higher than E2. ER_RBA=0 means no activity. All data was obtained from NCTRER (FDA National Center for toxicological Research Estrogen Receptor Binding Database) (Blair, Fang et al. 2000, Fang, Tong et al. 2001, Branham, Dial et al. 2002, Tong 2008).

Activity Score: For compounds with log(ER_RBA) values over 1E-5 in NCTRER database, the ER_RBA activity was spanned onto integer 20-100 activity range, making 100 the highest potency and 20 the lowest active potency.

Table 3.2. Source of hERα mutagenesis sites and known biological effects

Mutagenesis site	Source	Known Effect
Asp351Tyr	Breast cancer[1]	Increased estrogenicity triggered by antiestrogen Tamoxifen
Glu353Val	Breast cancer[2]	Decreased affinity to estrogen found with Gln mutation[7]
Met396Val	Breast cancer[3]	N/A
Leu525Ala	Artificial mutation[4]	Decreased estrogen binding and transcriptional activation
Asp538Gly	Breast Cancer[5]	Constitutively active receptor, Resistant to anti-estrogen treatment[6]

1. (Catherino, Wolf et al. 1995) 2. (Karnik, Kulkarni et al. 1994) 3. (Roodi, Bailey et al. 1995) 4. (Kirk, Karen et al. 1996) 5. (Forbes, Bhamra et al. 2008) 6. (Merenbakh-Lamin, Ben-Baruch et al. 2013) 7.(Ekena, Katzenellenbogen et al. 1998)

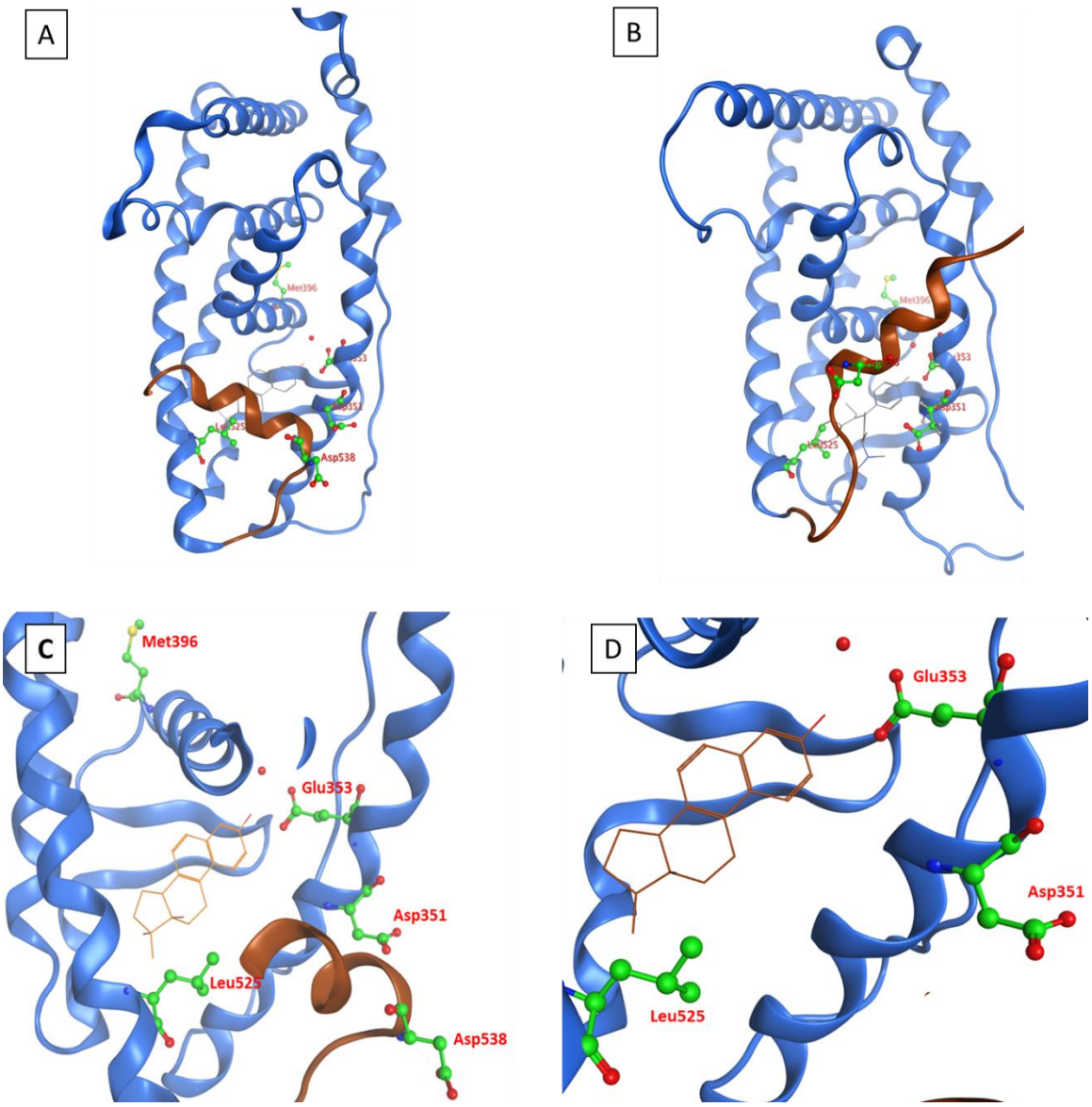


Fig 3.1. hERα ligand binding domain and mutation sites. Mutation sites are marked in green with residue tags in red. Helix-12 of the ligand binding domain is marked in brown. (A) agonist-bound form (PDB entry: 2B1Z), helix-12 is in closed position. (B) antagonist-bound form (PDB entry: 3ERT), helix-12 is in open position. (C&D) Close view of mutation sites on 2B1Z. Glu353 and Leu525 are inside the ligand binding pocket. Asp351 is on helix-3, outside the binding pocket, facing helix-12. Asp538 is on the end of helix-12. Met 396 is on the end of helix-5, outside the binding pocket.

Molecular dynamics simulation

The prepared 2B1Z (agonist form), 3ERT (antagonist form) wildtype and mutant protein structures without ligand were solvated in cube periodic box with 6 Å margin using the "Solvate" module in MOE. Each model was then energy-minimized to RMS gradient of 0.1 kcal/mol/$Å^2$ using CHARMM27 force field(Nicolas and Alexander 2000) in MOE with rigid water molecules constraint. Molecular dynamics simulations were performed using NAMD v2.9 (James, Rosemary et al. 2005) with CHARMM27 force field on Kraken supercomputer at National Institute for Computer Science, Oak Ridge, TN and Newton HPC at University of Tennessee, Knoxville. Protein models were heated from 0 to 300 K in 30 ps with heavy atom constraint of 0.5 Å, then equilibrated in Isothermal–isobaric (NPT) ensemble at 300 K, 101 kPa for 500 ps. Production simulation was performed at 300 K for 20 ns (50 ns for 2B1Z wildtype) in Canonical (NVT) ensemble with time step of 2 fs, fixed length for bonds with hydrogen, rigid water constraint, and Particle Mesh Ewald algorithm was applied to calculate electrostatic forces. Molecular dynamics (MD) trajectories were saved every 0.5 ps, producing 40000 frames for each 20 ns simulation.

Trajectory clustering and selection of MD conformation

Molecular dynamics trajectories were firstly trimmed down to protein backbone only form, and aligned using ProDy(Bakan, Meireles et al. 2011). Aligned trajectories were clustered based on RMSD (root-mean-square atom-to-atom distance) using the Clustering Tool plugin (Luis Gracia, Weill Cornell Medical College, New York, NY) featuring the quality-threshold clustering algorithm implemented in VMD(Humphrey, Dalke et al. 1996). Clustering was performed with 9 clusters specified, 1 Å cutoff threshold and step of 10 (use 1 of every 10 frames). N-terminal and C-terminal tail residues with large movement were excluded in clustering to minimize the unrelated

variance. One frame in each cluster was selected and based on the indices of the selected frames, original full-size frame was extracted from the untrimmed MD trajectory as PDB files using ProDy.

Ensemble docking

The selected MD snapshots of hERα ligand binding domain (LBD) structures 2B1Z and 3ERT wildtype and mutant, along with the starting structure for MD (referred as the crystal structures herein) (a total of 10 conformations for each molecule model) were used to dock the 29 estrogenic ligands listed in Table 3.1. For crystal structures, water molecules were removed except for one (water 8 in 2B1Z, water 2 in 3ERT) that is involved in the binding of various hydroxylated ligands through a hydrogen bond network with Glu353 and Arg354(Humphrey, Dalke et al. 1996, David, Yong et al. 1998, Shiau, Barstad et al. 1998). Similarly, a water molecule close to Glu353 and Arg354 was reserved in docking for snapshots selected from MD trajectories. Binding site for ligand docking was identified using the "Site Finder" module in MOE. For docking, ligand bonds were allowed to rotate during placement. Initial placement of ligands was conducted using Triangle Matcher method, with specified 1000 returned poses. The binding free energy of poses from initial placement were estimated by London dG scoring function and the top 30 poses were retained. The receptor atoms (within 6 Å distance from the ligands) and the ligands of retained poses were refined through force field (MMFF94x) energy minimization to 0.01 kcal/mol/Å RMS energy gradient, with side chains of the receptor pocket set as free to move. Final binding free energy scores were estimated by GBVI/WSA dG scoring function and the best scored pose was used for final comparison.

3.3 Results and Discussion

MD simulation of hERα LBD wildtype, mutants and ensemble docking results

MD simulation was performed for hERα ligand binding domain in the agonist-bound form (2B1Z) and antagonist bound form (3ERT), including both the wildtype and mutant structures. RMSD value for each simulation was calculated based on the protein backbone atoms and presented in Fig 3.2. All MD simulations were performed for 20 ns except for 2B1Z-wildtype, 50 ns simulation was conducted. The RMSD of 2B1Z structures were mostly within 2.5 Å, while 3ERT structures exhibited larger RMSD, with most showing 3.5 to 4 Å. The higher RMSD for 3ERT MD simulation is likely due to the different positioning of helix-12 in 3ERT that its helix-12 is in a more open position with more flexibility and space to move.

The trajectories of MD simulation were clustered based on the RMSD of backbone atoms. One snapshot was chosen from each of the 9 cluster to be used in the ensemble docking. Each of the 29 ligands listed in Table 3.1 was docked to 9 MD snapshots plus the starting structure, yielding 10 docking scores for each ligand/hER variant. The ensemble docking scores of hER wildtype and mutant are presented as 1) the average of all 10 dockings, 2) the best (lowest value) among all 10 dockings, and 3) the docking score of the starting structure for MD (crystal structure) (Supplemental Table 3.1).

Wildtype 2B1Z and 3ERT ensemble docking results and correlation with experimental binding affinity and transcriptional activity

Wild type 2B1Z and 3ERT ensemble docking results including the “ensemble average”, “ensemble best” and “crystal” structure docking scores are presented in Table 3.3 and Fig 3.2. To evaluate

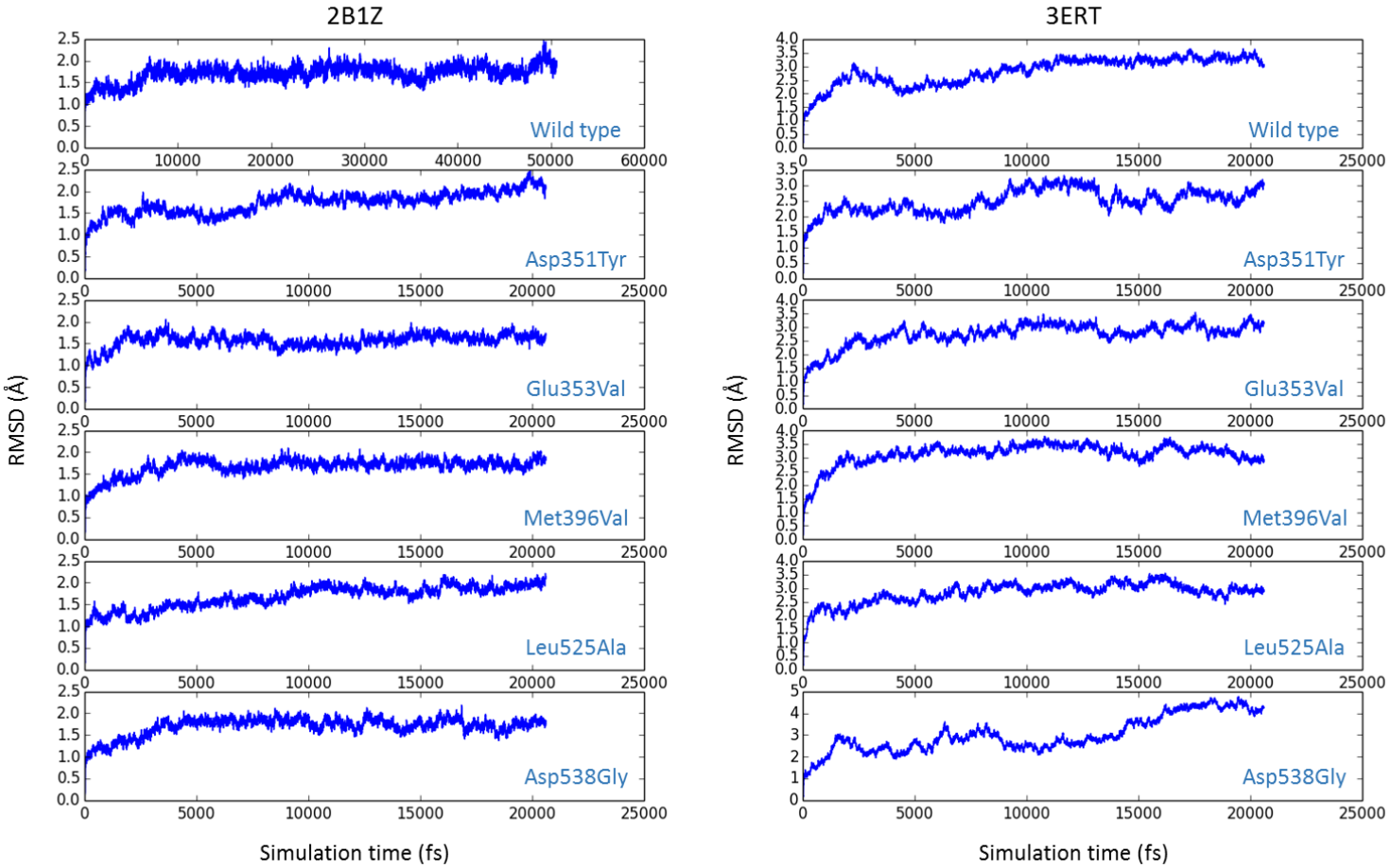


Fig 3.2. RMSD plots from MD simulation of 2B1Z and 3ERT wildtype and mutant structures.

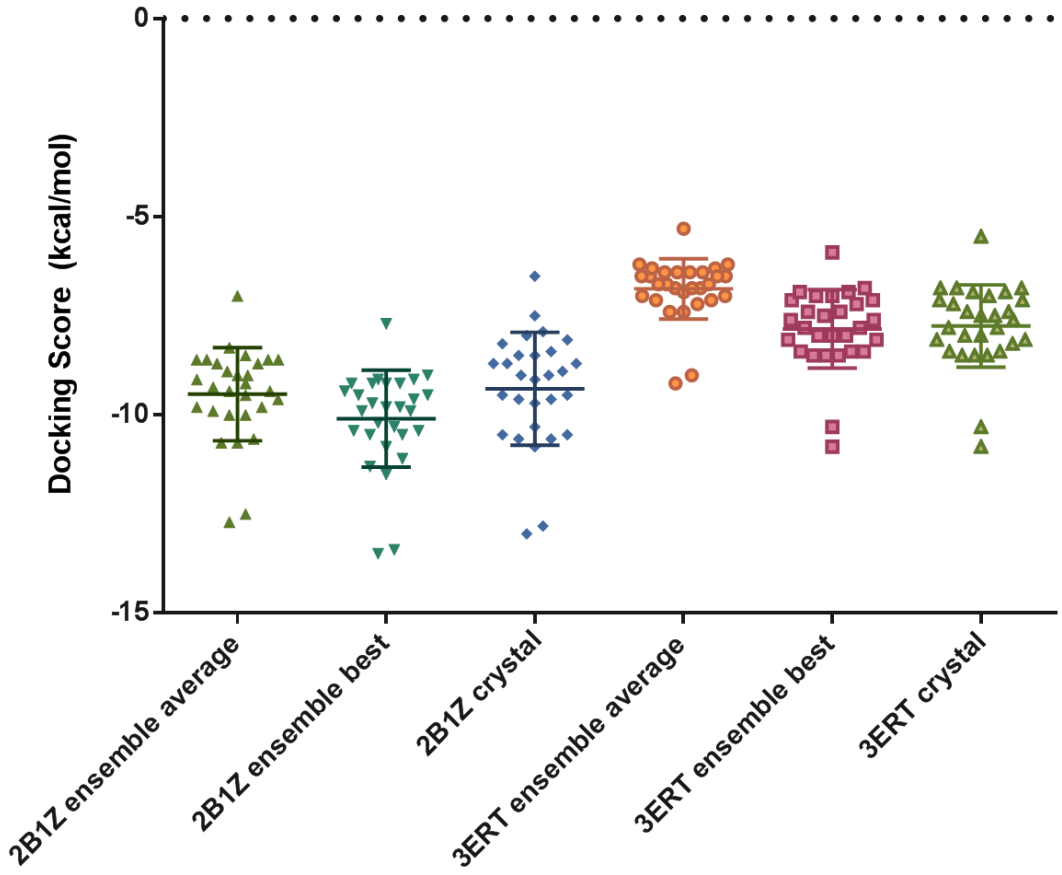


Fig 3.3. Column scatter plot for wildtype 2B1Z and 3ERT docking scores of 29 ligands. Bars represent mean and standard deviation.

the performance of ensemble docking in distinguishing ligands with different level of estrogenicity, the relative binding affinity from competitive estrogen receptor binding assay (ER_RBA), half-maximal effective concentration (EC50) from yeast bioluminescent estrogen reporter assay were obtained from previous studies(Tong 2008, Sanseverino, Eldridge et al. 2009) and listed in Table 3.3. Ligands in Table 3.3 are sorted by ER_RBA from the most potent to the least potent. Out of the 29 ligands, all except resveratrol have ER_RBA available, while 13 of them have EC50 estimated. The correlation between different docking scores, experimental relative binding affinity and EC50 values are presented in Table 3.4.

PDB structure 2B1Z and 3ERT represent the agonist and antagonist binding forms respectively for hERα LBD. The reason to include both conformations in the study was to examine which form is more suited for virtual docking screening of estrogenic compounds. In our study, docking scores obtained from 3ERT wildtype was larger than that of 2B1Z wildtype structures for any of the docked ligand, whether the comparison was based on the ensemble average, ensemble best or crystal docking scores (Table 3.3). When comparing the mean "crystal" docking scores of all ligands, 3ERT averaged -10.8 kcal/mol, while 2B1Z averaged -13.0 kcal/mol, 2.2 kcal/mol smaller than 3ERT. The mean of all ligands' "ensemble average" and "ensemble best" scores for 2B1Z and 3ERT also showed the similar difference of over 2 kcal/mol (Table 3.3 and Fig 3.3). Regarding how well the docking scores correlate with the experimental ER_RBA, 3ERT wildtype showed slightly less correlation than 2B1Z wildtype in "crystal" docking scores (-0.66 vs. -0.68), but significantly weaker correlation in "ensemble average" scores than 2B1Z (-0.49 vs. -0.61, Table 3.4). The difference between the docking score of 2B1Z and 3ERT can be attributed to 3ERT's Helix-12, which is in the open position, leaving ligands with a larger space compared to 2B1Z,

Table 3.3. Experimental relative binding affinity, EC50, ensemble docking average and best scores and crystal structure docking scores for wildtype 2B1Z and 3ERT structures

	ER_RBA	EC50 BLYES (M)	2B1Z ensemble average	2B1Z ensemble best	2B1Z crystal	3ERT ensemble average	3ERT ensemble best	3ERT crystal
diethylstilbestrol (DES)	3.98E+02	5.30E-12	-10.0	-10.4	-9.6	-6.8	-8.5	-8.5
ethynyl estradiol (EE2)	1.91E+02	2.50E-11	-9.9	-10.8	-10.5	-6.8	-8.4	-8.4
4-hydroxy-tamoxifen	1.74E+02	1.86E-09	-12.7	-13.4	-13.0	-9.2	-10.8	-10.8
17beta estradiol (E2)	1.00E+02	6.30E-10	-9.4	-9.9	-9.6	-6.7	-8.0	-8.0
alpha zearalanol	3.02E+01	-	-10.7	-11.1	-10.6	-7.4	-8.5	-8.5
estriol	9.77E+00	-	-9.8	-10.5	-10.5	-6.9	-8.1	-8.1
estrone	7.24E+00	6.40E-09	-9.4	-9.9	-9.5	-6.5	-7.8	-7.8
17alpha estradiol	3.09E+00	1.10E-08	-9.5	-10.3	-9.5	-6.7	-7.8	-7.8
mestranol	2.24E+00	-	-10.7	-11.5	-10.8	-7.2	-8.5	-8.5
zearalanone	2.09E+00	1.90E-06	-10.6	-11.3	-10.6	-7.4	-8.4	-8.2
tamoxifen	1.62E+00	-	-12.5	-13.5	-12.8	-9.0	-10.3	-10.3
coumestrol	8.91E-01	3.30E-08	-8.3	-9.1	-8.4	-6.2	-6.9	-6.8
genistein	4.36E-01	3.86E-06	-9.0	-9.8	-8.9	-6.3	-7.1	-7.1
norethynodrel	2.14E-01	1.38E-04	-10.0	-10.5	-10.3	-7.1	-8.4	-8.4
HPTE	2.51E-01	-	-9.3	-9.8	-9.1	-7.0	-8.1	-8.1
equol	1.51E-01	-	-9.0	-9.5	-8.7	-6.5	-7.6	-7.6
4',6-dihydroxyflavone	1.51E-01	-	-8.7	-9.2	-8.5	-6.4	-7.4	-7.4
bisphenol B	8.51E-02	-	-9.1	-9.7	-8.7	-6.8	-7.6	-7.5
nonylphenol	2.95E-02	1.70E-05	-9.8	-10.4	-9.0	-7.0	-7.5	-7.5
daidzein	2.20E-02	-	-8.9	-9.5	-8.7	-6.4	-7.2	-7.2
4-t-octylphenol	1.50E-02	-	-8.6	-9.0	-8.1	-6.5	-7.1	-6.9
bisphenol A (BPA)	7.80E-03	-	-8.7	-9.1	-8.5	-6.5	-7.4	-7.1
4'-hydroxychalcone	3.70E-03	-	-8.6	-9.2	-8.0	-6.3	-7.0	-7.0
4'-hydroxy flavanone	2.23E-03	-	-8.6	-9.2	-8.2	-6.4	-6.8	-6.8
5alpha dihydrotestosterone	1.30E-03	3.71E-06	-9.6	-10.2	-9.7	-6.7	-8.0	-8.0
o,p'-DDT	1.41E-03	-	-9.2	-9.6	-9.0	-7.1	-8.0	-7.4

Table 3.3 Continued

	ER_RBA	EC50 BLYES (M)	2B1Z ensemble average	2B1Z ensemble best	2B1Z crystal	3ERT ensemble average	3ERT ensemble best	3ERT crystal
chalcone	1.51E-03	-	-8.6	-9.2	-7.5	-6.2	-6.9	-6.9
ethyl 4-hydroxybenzoate	6.00E-04	1.40E-06	-7.0	-7.7	-6.5	-5.3	-5.9	-5.5
resveratrol	-	-	-8.5	-9.4	-7.9	-6.4	-7.0	-6.8
min	6.00E-04	5.30E-12	-12.7	-13.5	-13.0	-9.2	-10.8	-10.8
max	3.98E+02	1.38E-04	-7.0	-7.7	-6.5	-5.3	-5.9	-5.5
mean	3.29E+01	1.28E-05	-9.5	-10.1	-9.3	-6.8	-7.8	-7.8
stdev	8.57E+01	3.64E-05	1.2	1.2	1.4	0.8	1.0	1.0

-: no data available.

EC50_BLYES: half-maximal effective concentration (mol/L) previously determined by bioluminescent yeast estrogen reporter(Sanseverino, Eldridge et al. 2009).

2B1Z and 3ERT are the two crystal structure of hERα ligand binding domain representing the agonist-bound and antagonist bound conformation respectively. Ensemble average is the averaged docking score (predicted binding affinity from GBVI/WSA dG scoring function, kcal/mol) from all ensemble docking. Ensemble best is the lowest docking score from all ensemble docking. Crystal is the docking score using the crystal structure of the receptor protein.

Table 3.4. Pearson's correlation between experimental binding affinity and EC50, ensemble docking average and best scores and crystal structure docking scores

	logER_RBA	logEC50 (BLYES)	2B1Z ensemble average	2B1Z ensemble best	2B1Z crystal	3ERT ensemble average	3ERT ensemble best	3ERT crystal
logER_RBA	1.00							
logEC50 (BLYES)	-0.81*	1.00						
2B1Z ensemble average	-0.61*	0.21	1.00					
2B1Z ensemble best	-0.61*	0.21	0.99*	1.00				
2B1Z crystal	-0.68*	0.26	0.97*	0.97*	1.00			
3ERT ensemble average	-0.49*	0.15	0.96*	0.95*	0.92*	1.00		
3ERT ensemble best	-0.63*	0.34	0.97*	0.95*	0.96*	0.96*	1.00	
3ERT crystal	-0.66*	0.35	0.97*	0.96*	0.96*	0.94*	0.99*	1.00

*: $p<0.05$

logER_RBA: log trasnformed estrogen receptor relative binding affinity measured by rat uterine cytosol estrogen receptor competitive receptor binding assay, obtained from NCTRER (FDA National Center for toxicological Research Estrogen Receptor Binding Database) (Blair, Fang et al. 2000, Fang, Tong et al. 2001, Branham, Dial et al. 2002, Tong 2008).

logEC50 (BLYES): log transformed half-maximal effective concentration previously determined by bioluminescent yeast estrogen reporter(Sanseverino, Eldridge et al. 2009).

hence yield lower predicted binding affinity. The majority of the 29 ligands except tamoxifen and 4-hydroxy-tamoxifen are agonists of hER, and 3ERT yielded weaker predicted binding affinity and slightly poorer correlation with experimental ER_RBA as compared to 2B1Z. Consequently, further downstream data analysis was primarily focused on 2B1Z derived results. The data for relative comparison to 3ERT is available for further use (see Supplemental Table 3.1).

ER_RBA is the experimental binding affinity obtained through competitive ligand binding assay in the presence of natural estrogen E2. Out of the 29 tested ligands, 28 of them have ER_RBA data available. These ligands ranged from strong to medium ER binder according to the ER_RBA based annotation in NCTRER database, and their ER_RBA spread from 6E-4 to 3.98E+2, a range of approximately six magnitude (Table 3.1). According to ER_RBA, the strongest ER binder is DES while the weakest binder is ethyl 4-hydroxybenzoate. In our docking results of 2B1Z wildtype, ethyl 4-hydroxybenzoate was indeed predicted to be the weakest binder, based on either "ensemble average", "ensemble best" or "crystal" scores. Yet the predicted strongest binder was not DES, but tamoxifen. To further assess the performance of the docking scheme, correlations between virtual docking scores and experimental binding and yeast bioassay results were analyzed (Table 3.4). logER_RBA had moderate correlation with either 2B1Z "ensemble average", "ensemble best" or "crystal". "Ensemble best" and "ensemble average" yielded the same coefficient of -0.61, while "crystal" yielded slightly higher coefficient than "ensemble average" and "ensemble best" (-0.68 vs. -0.61). These results suggest that for wildtype 2B1Z docking, crystal structure actually provided slightly better performance in distinguishing the binding affinity of the tested 29 ligands. Experimental ligand potency data evaluated by yeast estrogen bioluminescent reporter BLYES(Sanseverino, Eldridge et al. 2009) in the form of EC50 were also

available for 13 of the 29 ligands. The cited EC50 data represent the potency of ligands in activating hER and triggering the reporter bioluminescent genes, while ER_RBA focused on the ligand binding process without considering the subsequent gene transactivation. Despite the difference, the two experimental data, EC50 of the 13 ligands, agrees well with their ER_RBA, in a strong correlation of -0.81 (Table 3.4). However there were no statistically significant correlations found between EC50 and 2B1Z wildtype docking scores. Collectively, the results from correlation analysis suggest that the ability of the docking scheme with 2B1Z to distinguish between strong to medium hER binders is moderate at best. Although the ligand binding domain of hERα demonstrated very good selectivity in previous virtual screening studies, these success results were evaluated on its ability to distinguish between agonist and decoys with over 1000 ligands been screened(Schapira, Abagyan et al. 2003, Harris, Eldridge et al. 2014). In comparison, our study only docked a small number of 29 known hERα binders without inclusion of decoys. The fact that all docked ligands have medium to strong hERα binding potency and the small scale of screening could be the reason for the only moderate correlation between our docking scores and the experimental binding affinity.

In order to make comparison between mutant and wildtype docking, one of the three different types of docking scores needs to be determined. For several considerations as discussed below, “ensemble average” was eventually selected over “ensemble best” and “crystal” scores. Firstly, “crystal” scores was excluded since for the mutant hER receptors, there was no real “crystal” structure. As the purpose of MD simulation of the mutant hER receptor was to explore potential structural changes as the result of mutations, using the “crystal” score would defeat this purpose. Additionally, to support the use of “ensemble average”, Glu353Val mutant docking scores were

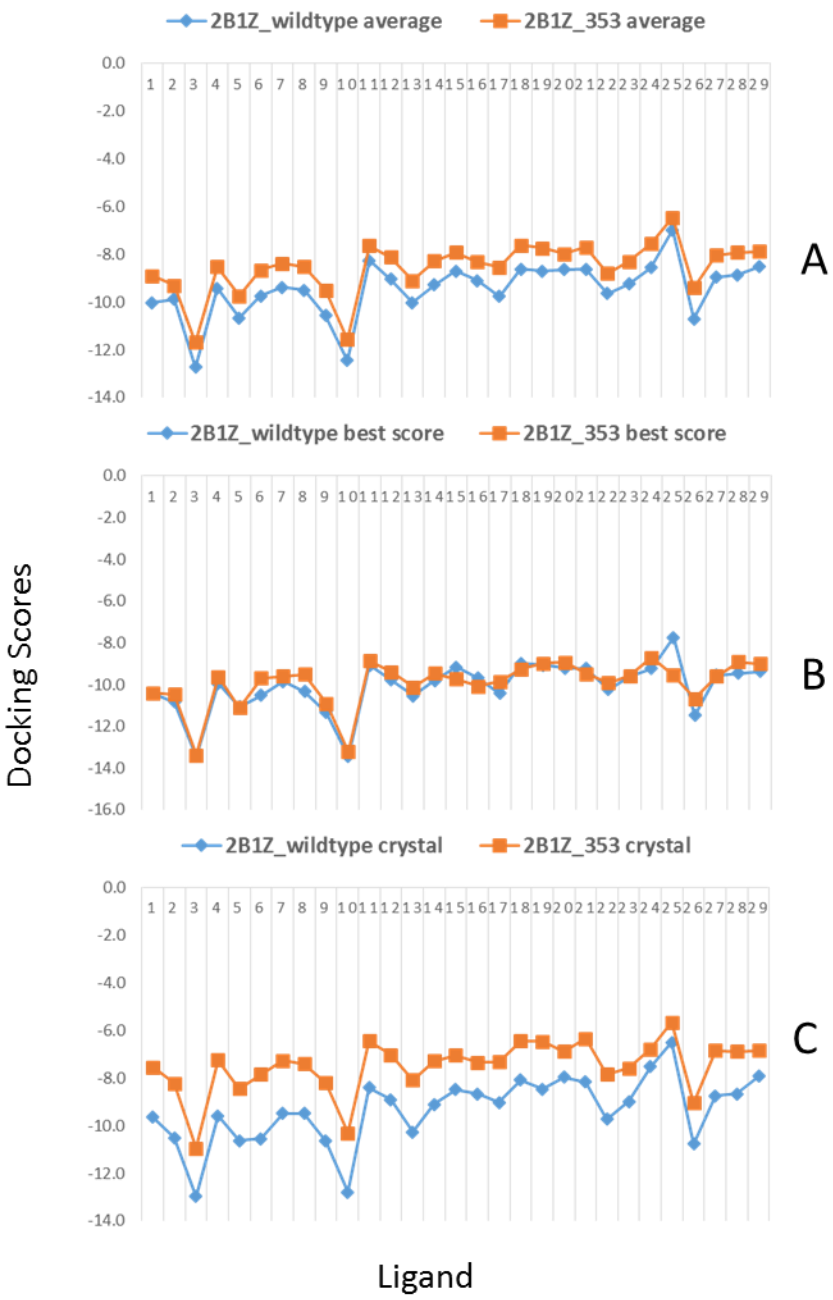


Fig. 3.4. Comparison of docking scores for 2B1Z wildtype and Glu353Val mutant. (A) Ensemble average docking scores, (B) Ensemble best docking scores, (C) Crystal docking scores.

Table 3.5. 2B1Z wildtype and Glu353Val mutant docking scores comparison

	ensemble average		ensemble best		crystal	
	2B1Z_wildtype	2B1Z_353	2B1Z_wildtype	2B1Z_353	2B1Z_wildtype	2B1Z_353
diethylstilbestrol (DES)	-10.0	-8.9	-10.4	-10.4	-9.6	-7.5
ethynyl estradiol	-9.9	-9.3	-10.8	-10.5	-10.5	-8.2
4-hydroxy-tamoxifen	-12.7	-11.7	-13.4	-13.4	-13.0	-10.9
17beta estradiol (E2)	-9.4	-8.5	-9.9	-9.6	-9.6	-7.2
alpha zearalanol	-10.7	-9.7	-11.1	-11.1	-10.6	-8.4
estriol	-9.8	-8.7	-10.5	-9.7	-10.5	-7.8
estrone	-9.4	-8.4	-9.9	-9.6	-9.5	-7.3
17alpha estradiol	-9.5	-8.5	-10.3	-9.5	-9.5	-7.4
zearalanone	-10.6	-9.5	-11.3	-10.9	-10.6	-8.2
tamoxifen	-12.5	-11.6	-13.5	-13.2	-12.8	-10.3
coumestrol	-8.3	-7.6	-9.1	-8.9	-8.4	-6.4
genistein	-9.0	-8.1	-9.8	-9.4	-8.9	-7.0
norethynodrel	-10.0	-9.1	-10.5	-10.1	-10.3	-8.1
HPTE	-9.3	-8.3	-9.8	-9.5	-9.1	-7.3
4',6-dihydroxyflavone	-8.7	-7.9	-9.2	-9.7	-8.5	-7.0
bisphenol B	-9.1	-8.3	-9.7	-10.1	-8.7	-7.3
nonylphenol	-9.8	-8.5	-10.4	-9.9	-9.0	-7.3
4-t-octylphenol	-8.6	-7.6	-9.0	-9.3	-8.1	-6.4
bisphenol A	-8.7	-7.7	-9.1	-9.0	-8.5	-6.4
4'-hydroxychalcone	-8.6	-8.0	-9.2	-8.9	-8.0	-6.9
4'-hydroxy flavanone	-8.6	-7.7	-9.2	-9.5	-8.2	-6.3
5alpha dihydrotestosterone	-9.6	-8.8	-10.2	-9.9	-9.7	-7.8
o,p'-DDT	-9.2	-8.3	-9.6	-9.6	-9.0	-7.6
chalcone	-8.6	-7.6	-9.2	-8.7	-7.5	-6.8
ethyl 4-hydroxybenzoate	-7.0	-6.4	-7.7	-9.6	-6.5	-5.7
mestranol	-10.7	-9.4	-11.5	-10.7	-10.8	-9.0
equol	-9.0	-8.0	-9.5	-9.6	-8.7	-6.8
daidzein	-8.9	-7.9	-9.5	-8.9	-8.7	-6.9
resveratrol	-8.5	-7.9	-9.4	-9.0	-7.9	-6.8
min	-12.7	-11.7	-13.5	-13.4	-13.0	-10.9
max	-7.0	-6.4	-7.7	-8.7	-6.5	-5.7
mean	-9.5	-8.6	-10.1	-9.9	-9.3	-7.5
stdev	1.2	1.1	1.2	1.1	1.4	1.1

used to demonstrate the suitability of “ensemble average” score in distinguishing the mutation effect (Table 3.5, Fig 3.4). When comparing the “ensemble average” and “ensemble best” scores of 2B1Z wildtype and 2B1Z_353 mutant, “ensemble average” better reflected the expected weaker binding as a result of Glu353Val mutation. Glu353Val mutant included in this study can be viewed as a positive control for predicted weaker binding, as Glu353 residue is the essential residue in hER ligand binding pocket. Through its charged side chain, Glu353 supports the anchoring and correct positioning of many ligands including the natural estrogen E2 and ligands with similarly positioned hydroxyl group. When Glu353 was mutated into Val, all ligands rely on the electrostatic interaction with Glu353 would suffer from significantly weaker binding. From Fig.3.4, it’s clear that “ensemble average” was able to differentiate 2B1Z wildtype and Glu353Val mutant while “ensemble best” failed to distinguish. Therefore “ensemble average” was eventually chosen as the score for wildtype and mutant binding affinity comparison.

Comparison of 2B1Z mutant and wildtype docking results, effect of mutation on ligand docking scores

The “ensemble average” docking scores for the five hER mutants was compared with the 2B1Z wildtype and presented as percentage of changes (Table 3.6). A negative percentage means smaller predicted binding affinity compared to the wildtype. Docking scores are also plotted as column scatter plot, as presented in Fig 3.5.

When examining the docking scores of all 29 ligands as a whole, all five mutants demonstrated various degrees of reduction in predicted binding affinity compared to the wildtype (Fig3.5). For docking scores, the larger negative value has the higher predicted binding affinity. Among the

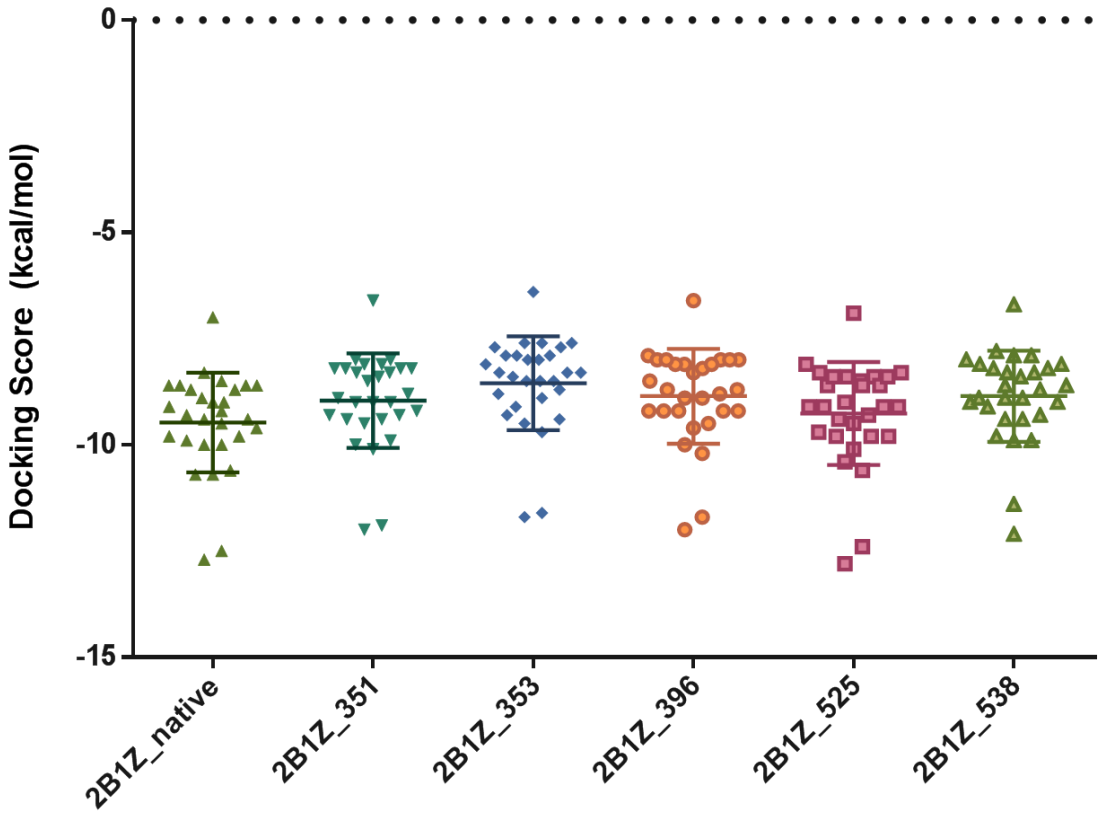


Fig 3.5. Column scatter plot of ensemble average docking scores of 2B1Z wildtype and mutants for all 29 ligands. Bars represent mean and standard deviation.

Table 3.6. Percentage changes of ensemble average docking scores of 2B1Z mutants in comparison to the wildtype

	2B1Z_351	2B1Z_353	2B1Z_396	2B1Z_525	2B1Z_538
diethylstilbestrol (DES)	-7%	-11%	-5%	-3%	-7%
ethynyl estradiol	-4%	-6%	-7%	-1%	-5%
4-hydroxy-tamoxifen	-5%	-8%	-6%	0%	-5%
17beta estradiol (E2)	-5%	-10%	-6%	-3%	-5%
alpha zearalanol	-5%	-9%	-4%	-1%	-7%
estriol	-5%	-11%	-5%	-3%	-8%
estrone	-4%	-11%	-7%	-4%	-8%
17alpha estradiol	-6%	-10%	-4%	-4%	-6%
zearalanone	-6%	-10%	-5%	-2%	-6%
tamoxifen	-5%	-7%	-6%	-1%	-8%
coumestrol	-3%	-8%	-3%	1%	-5%
genistein	-7%	-10%	-9%	-5%	-8%
norethynodrel	-6%	-9%	-8%	-2%	-7%
HPTE	-5%	-11%	-4%	-2%	-6%
4',6-dihydroxyflavone	-6%	-9%	-9%	-3%	-5%
bisphenol B	-7%	-9%	-6%	-1%	-5%
nonylphenol	-5%	-13%	-6%	-1%	-8%
4-t-octylphenol	-5%	-12%	-6%	-3%	-6%
bisphenol A	-6%	-11%	-6%	-2%	-6%
4'-hydroxychalcone	-4%	-8%	-8%	0%	-4%
4'-hydroxy flavanone	-6%	-11%	-7%	-4%	-6%
5alpha dihydrotestosterone	-4%	-9%	-10%	-2%	-5%
o,p'-DDT	-4%	-10%	-5%	0%	-3%
chalcone	-6%	-12%	-5%	-2%	-9%
ethyl 4-hydroxybenzoate	-5%	-8%	-6%	-1%	-3%
mestranol	-7%	-12%	-11%	-6%	-9%
equol	-8%	-10%	-9%	-4%	-6%
daidzein	-7%	-11%	-9%	-6%	-10%
resveratrol	-5%	-7%	-7%	-5%	-7%
Max	-8%	-13%	-11%	-6%	-10%
Min	-3%	-6%	-3%	1%	-3%
Mean	-5%	-10%	-7%	-2%	-6%
Stdev	1%	2%	2%	1%	2%

five mutants, 2B1Z_353 mutant yielded the largest decrease in predicted binding affinity; on average, docking scores of 2B1Z_353 for all 29 ligands were 10% higher than the wildtype (Table 3.6). On the other hand, 2B1Z_525 mutant produced the smallest decrease in docking scores; the average percentage of change for all 29 ligands was only -2%. The other three mutants, 2B1Z_351, 2B1Z_396 and 2B1Z_538 all demonstrated modest reduction of predicted binding, with the average percentage of change being -5%, -7% and -6% respectively. The difference in docking scores between the wildtype and mutants for individual ligand varied as well. 2B1Z_353 yielded the steepest decreases, with percentage of change ranged from -13% to -6%. For 2B1Z_351, 2B1Z_396, 2B1Z_538, percentage of decrease ranged from -8% to -3%, -11% to -3% and -10% to -3%, respectively. 2B1Z_525, however, was the only mutant that showed no change or an increase of binding affinity for several ligands; its percentage of change ranged from -6% to 1%. Overall, ensemble docking simulation predicted decrease of binding affinity for nearly all 29 ligands when the tested five hERα mutations occur. In the five hER mutations, Glu353Val was predicted to yield the highest reduction of binding affinity, while Leu525Ala was predicted to yield the least reduction, with some ligands showed no change or slight increase.

To interpret the predicted results, it is necessary to understand the position, and potential role of each individual mutated residue of the receptor. Among the five mutation sites, only Glu353 and Leu525 are considered inside the binding pocket that can directly interact with ligands, while Asp351 and Met396 and Asp538 are in the vicinity of the binding pocket (Fig 3.1). Glu353Val and Leu525Ala mutations can be better evaluated through virtual docking as the mutations create more drastic changes directly inside the binding cavity that are easier to be reflected on the docking scores. Glu353 residue features negatively charged side chain that is responsible for

critical electrostatic interaction with ligands with hydroxyl group. Mutating Glu353 to Val eliminates this critical ligand-receptor electrostatic interaction. Such drastic change was clearly reflected on the noticeable shift of 2B1Z_353 docking scores. Leu525 is located at the opening end of the binding pocket, and helps shape the binding cavity through its hydrophobic side chain. Mutating Leu525 to Ala displaces the long hydrophobic side chain, creating opener binding cavity. Depending on the structure of a ligand, a more open binding cavity could either benefit or negatively affect the binding affinity. The docking of 29 ligands to Leu525Ala seems to support this perception, as the prediction posted mostly small decreases of binding affinity, while several remained the same or had slightly increase. Although Asp351 residue is close to Glu353, its side chain stretches outwards of the ligand binding pocket (Fig 3.1). Previous studies have reported that Asp351 residue interacts with antagonists such as tamoxifen (Levenson, Catherino et al. 1997, Levenson and Jordan 1998). However, whether the Asp351Tyr mutation can cause a structural change in the binding pocket that affects the binding of other agonists was unknown. In our study, the ensemble docking of 2B1Z_351, 2B1Z_396 and 2B1Z_538 predicted decreases of binding affinity as the result of these mutations for all 29 ligands. Since Asp351, Met396, Glu538 are outside the binding pocket, the predicted changes of binding affinity through ensemble docking of MD sampled conformations indicate the occurrence of structural transition during MD simulation that made the binding pocket of the receptor less fit to the ligands. Nevertheless, as the difference between 2B1Z wildtype and mutant docking scores was very modest, the accuracy of the predictions warrants validation through experimental mutagenesis study.

3.4 Conclusion

In this study, ensemble docking of selected MD conformations of wildtype hERα ligand binding domain showed moderate correlation with previous experimental binding assay data, suggesting a relatively good performance of the docking scheme, especially considering the evaluated ligands were strong to medium ER binders. When comparing the 2B1Z derived docking results between hERα wildtype and the five mutants, ensemble docking simulation predicted decrease of binding affinity for nearly all 29 ligands when the tested five hERα mutations occur. Among the five hER mutations, Glu353Val was predicted to yield the highest reduction of binding affinity, while Leu525Ala was predicted to yield the least reduction, with some ligands demonstrated no change or slightly increase in binding affinity. However, as the difference between 2B1Z wildtype and mutant docking scores was very modest, the accuracy of the predictions requires further validation through experimental mutagenesis study.

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Chapter 4. Evaluating the Effects of Human Estrogen Receptor Mutations in Response to environmental EDCs using Yeast Bioluminescent Estrogen Reporter

Abstract

In this chapter, the yeast estrogen bioreporters were constructed to test the biological role of human estrogen receptor alpha (hERα) mutation in altering ligand binding and transcriptional response to environmental chemicals with a wide variety of structure and origin. In order to provide a comparable platform, five mutated hERα genes, each containing a missense mutation close to the ligand binding pocket, along with the wild type, were integrated separately into the same location of yeast genome as single copy gene, and expressed by the same promoter and terminator. Each hER mutant carrying yeast strain was also transformed with a bioluminescent reporter plasmid which contains the estrogen response element controlled promoter, allowing the evaluation of transcriptional activity triggered by ligand-receptor interaction. A total of 12 EDCs including the natural estrogen 17β-estradiol (E2), synthetic hormone, plasticizer, phytoestrogen and industrial compound were evaluated with the wildtype and mutant hER yeast bioreporters.

4.1 Introduction

hERα is a nuclear receptor that can regulate gene expression when activated through ligand binding and subsequent conformational change. hERα plays critical roles in the regulation of hormonal responses and is highly related to critical diseases including breast cancer, osteoporosis etc. The ligand-dependent transcriptional activation of hER requires other essential coregulators and heterologous expression of the human estrogen receptor with fully functional transcriptional activity relies on the presence of proper coregulators in the selected hosts. Human estrogen receptor alpha were find fully functional as transcriptional activator in *S. cerevisiae,* but not in

prokaryotic organisms such as *E.coli*(Metzger, White et al. 1988). As the most basic eukaryotic organism, *Saccharomyces cerevisiae* is a well characterized host with efficient molecular manipulation tools available, and has been widely used for mammalian gene expression. The fact that human estrogen receptor can maintain full transcriptional activity in *S. cerevisiae* lead to many uses of it for hER structural and functional studies. Particularly, manipulating and maintaining yeast cell is much easier, quicker and lower cost compared to working with mammalian cells.

The aim of this present study was to take advantage of the yeast estrogen bioreporter system as a platform to test the biological role of human estrogen receptor alpha (hERα) mutation in altering ligand binding and transcriptional response to environmental chemicals with a wide variety of structure and origin. In order to provide a comparable platform, five mutated hERα genes, each containing a missense mutation close to the ligand binding pocket, along with the wild type, were integrated separately into the same location of yeast genome as single copy gene, and expressed by the same promoter and terminator. Each hER mutant carrying yeast strain was also transformed with a bioluminescent reporter plasmid which contains the estrogen response element controlled promoter, allowing the evaluation of transcriptional activity triggered by ligand-receptor interaction. These constructed yeast reporters provided the opportunity to quickly evaluate the transcriptional activity changes when certain mutations occurred on hERα. A total of 12 EDCs including the natural estrogen 17β-estradiol (E2), synthetic hormone, plasticizer, phytoestrogen and industrial compound were evaluated with the wildtype and mutant hER yeast bioreporters.

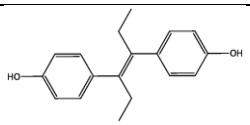
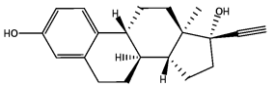
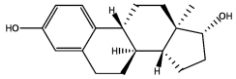
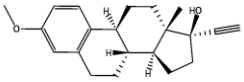
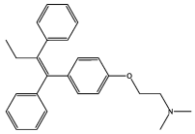
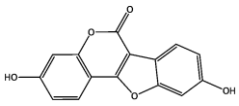
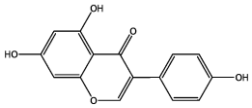
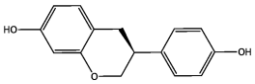
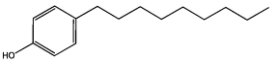
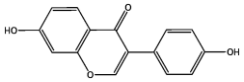
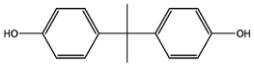
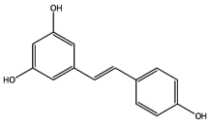
4.2 Constructing New BLYES and Variant Reporters Carrying hER Mutations and Optimizing the Chemical Testing Protocol

4.2.1 Methods and Materials

Chemicals, strains, medium and reagents

All EDCs used in the study are listed in Table 4.1. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). *Saccharomyces cerevisiae* BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) was purchased from the American Type Culture Collection (ATCC 201389™). YPD medium (yeast extract 10 g/L, peptone 20g/L, glucose 20 g/L, 18 g/L of agar for plate) was used for growth and maintain of untransformed yeast strain. For selection of transformant carrying KanMX4 selective marker, 300 mg/L effective concentration of antibiotic G418 was added to the YPD medium. Yeast synthetic drop medium without uracil (YSD -ura) was used for selecting and maintaining yeast strains harboring plasmid with *ura3* selective marker. YSD medium was made according to the manufacturer's instruction, and contains: 1) 1.92 g/L yeast synthetic drop-out media supplements (Sigma-Aldrich, St. Louis, MO, cat# Y1501), 2) 6.7 g/L yeast nitrogen base without amino acids (Sigma-Aldrich, St. Louis, MO, cat# Y0626), 3) 20g/L dextrose. Because ammonium sulfate contained in yeast nitrogen base can disrupt the function of G418, the minimal medium for *KanMX4* and *ura3* selection was made with: 1) 1.92 g/L yeast synthetic drop-out media supplements (Sigma-Aldrich, St. Louis, MO, cat# Y1501), 2) 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate (Sigma-Aldrich, St. Louis, MO, cat# Y1251), 3) 20g/L dextrose, 4) 300 mg/L effective concentration of G418. This medium is only suitable for maintaining strains, not suitable for initial yeast transformant selection due to very low yield of colonies.

Table 4.1 Estrogenic compounds structures and relative binding affinity

	Structure	CAS Registry Number	ER_RBA	Activity Category	Category
diethylstilbestrol (DES)		56-53-1	398.10001	active strong	Pharmaceutical, synthetic hormone
ethynyl estradiol		57-63-6	190.5	active strong	Pharmaceutical, synthetic hormone
17β estradiol (E2)		50-28-2	100	active strong	Natural hormone
mestranol		72-33-3	2.24	active strong	Pharmaceutical, synthetic hormone
tamoxifen citrate		54965-24-1	1.62	active strong	Pharmaceutical, anti-estrogen
coumestrol		479-13-0	0.89099997	active medium	Phytoestrogen
genistein		446-72-0	0.43599999	active medium	Phytoestrogen
S-equol		531-95-3	0.15099999	active medium	Phytoestrogen
4-nonylphenol		25154-52-3	0.0295	active medium	Industrial compound
daidzein		486-66-8	0.022	active medium	Phytoestrogen
bisphenol A		80-05-7	0.0077999998	active weak	Plasticizer
Resveratrol		501-36-0	na	na	Phytoestrogen

ER_RBA, activity score and activity category are obtained from NCTRER database (FDA National Center for toxicological Research Estrogen Receptor Binding Database)(Tong 2008).

ER_RBA: estrogen receptor binding affinity (relative to 17β-estradiol) measured by rat uterine cytosol estrogen receptor competitive receptor binding assay. Natural estrogen E2 has ER_RBA = 100. ER_RBA > 100 means ligand's binding affinity is higher than E2. ER_RBA=0 means no activity. All data was obtained from NCTRER (FDA National Center for toxicological Research Estrogen Receptor Binding Database) (Blair, Fang et al. 2000, Fang, Tong et al. 2001, Branham, Dial et al. 2002, Tong 2008).

Activity Score: For compounds with log(ER_RBA) values over 1E-5 in NCTRER database, the ER_RBA activity was spanned onto integer 20-100 activity range, making 100 the highest potency and 20 the lowest active potency.

na: no data available.

Chemically competent *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) was used as the host for transforming constructed plasmids following the manufacturer's protocol. Growth and maintenance of *E. coli* TOP10 was on Luria-Bertani medium at 37°C and 100 µg/ml of ampicillin was added when ampicillin-resistant plasmid selection was needed. Plasmid isolation was performed using Zippy Plasmid Miniprep Kit (Zymo Research, Irvine, CA). T4 ligase and all restriction endonucleases were purchased from Promega (Madison, WI). All PCR reactions were carried out with Q5 High-Fidelity DNA Polymerase (New England Biolabs, MA). All oligonucleotides used in this section are listed in Table 4.2.

Plasmids

pHO-Poly-KanMX4-HO was a gift from David Stillman (Addgene plasmid # 51662)(Voth, Richards et al. 2001). p424GPD was provided by Melinda Hauser (University of Tennessee, Knoxville)(Dominik, Rolf et al. 1995). The base plasmid pEREMEL1-Lux was a gift from Tingting Xu (Center for Environmental Biotechnology, University of Tennessee). pEREMEL1-Lux is a self-replicative plasmid featuring an estrogen response element (ERE) fused MEL1 promoter, which initiates transcription when bounded by activated estrogen receptor protein. Viral 2A elements were used to link and co-express six essential genes (*frp* and *luxCDABE*) for bioluminescence production under the control of ERE fused MEL1 promoter (Fig 4.1)(Xu, Ripp et al. 2014).

Cloning hERα gene from *Saccharomyces cerevisiae* BLYES strain

S. cerevisiae BLYES strain carries a genomic integrated human estrogen receptor alpha gene(Sanseverino, Gupta et al. 2005). However, from previous literature there was no information regarding the actual sequence of this integrated gene. For comparison purpose, the

Table 4.2 Primers and oligonucleotides used in bioreporter constructions.

Primer Name	Sequence (5'->3')	Template	Description
HO_F	ttaattatcctgggcacgag	pHO-424GPD-hER	Amplify the DNA fragment for single-copy genomic integratgration of hER expression cassette
HO_R	ACTGTAAGATTCCGCCACAT		
HOPoly_cyc1t_GPD_F	gtacgctgcaggtcgacggatccccGGTAC CGGCCGCAAATTA	p424GPD	Amplify the promoter-terminator (GPD-CYC1) region of p424GPD
HOPoly_cyc1t_GPD_R	agatctggcgcgccttaattaacccTCGAG TTTATCATTATCAATACTGCC		
shER_gib_HO_F	cgacggatctagaactagtgATGACTATG ACATTACACAC	hER-wt	Amplify hER-wt gene, allow insertion of the amplified gene to the p424GPD or pHO-GPDp-CYC1t-KanMX4-HO plasmid's polylinker site.
shER_gib_HO_R	tatcgataagcttgatatcgTTATTAGACT GTTGCTGGG		
ohER_gib_HO_F	cgacggatctagaactagtgATGACCATG ACCCTCCACAC	hER-y	Amplify hER-y gene, allow insertion to p424GPD or pHO-GPDp-CYC1t-KanMX4-HO plasmid's polylinker site.
ohER_gib_HO_R	tatcgataagcttgatatcgTTATTAACCG TGGCAGGGAA		
ohER_F_BamHI	gatgcaGGATCCATGACCATGACCCT CCACACCAA	hER-y	Amplify hER-y from *S. cerevisiae* BLYES strain, contain restriction site, and allow ligation to p424GPD.
ohER_R_EcoRI	cgatgcGAATTCACCGTGGCAGGGA AACCCTCT		
P424_BamHI_F	CACTAGTTCTAGATCCGTC	p424GPD or pHO-GPDp-CYC1t-KanMX4-HO	Amplify and linearize the p424GPD plasmid ending with BamHI and EcoRI sites.
P424_EcoRI_R	CGATATCAAGCTTATCGATA		
pERE_Up_pro	aagcttggcgtaatcatggt	pEREMEL1-lux	Amplify/linearize the pERMEL1-lux plasmid without the EREMEL1 fragment.
pERE_Down_pro	ggtaccATGGGCACCAAGA		
ERECYC1_F+pERE	ctatgaccatgattacgccaagcttgagctCA AAGTCAGGTCA	ERECYC1	Amplify ERECYC1 sequence
ERECYC1_R+pERE	TGATCTTCTTGGTGCCCATggtaccA TTAGTGTGTGTATTTGTGTTTGC		
S20_pERE_F	gtctgTTTGGTAGCGGCTGCTTTATat ccgatgataagctgtcaa	pEREMEL1-lux	Amplify pEREMEL1-Lux without the 2micron fragment
S20_pERE_R	attatATGATATAAAGGCGCCTGGCt gaagcacagatgcttcgtt		
20-up_F	GCCAGGCGCCTTTATATCAT	*S. cerevisiae* genomic DNA	Amplify S20 fragment from yeast genome
20_down_R	ATAAAGCAGCCGCTACCAAA		
20-up_R	TTTGCGAAACCCTATGCTCT	pS20-EREMEL1-lux	Amplify and linearize p20-pEREMEL1-Lux plasmid for yeast genomic integration.
20-down_F	AATGGAAGGTCGGGATGAG		

S. cerevisiae BLYES: yeast bioluminescent estrogen reporter previous developed(Sanseverino, Gupta et al. 2005).
hER-wt: wildtype 595 amino acids human estrogen alpha
hER-y: human estrogen receptor alpha in *S. cerevisiae* BLYES strain(Sanseverino, Gupta et al. 2005).

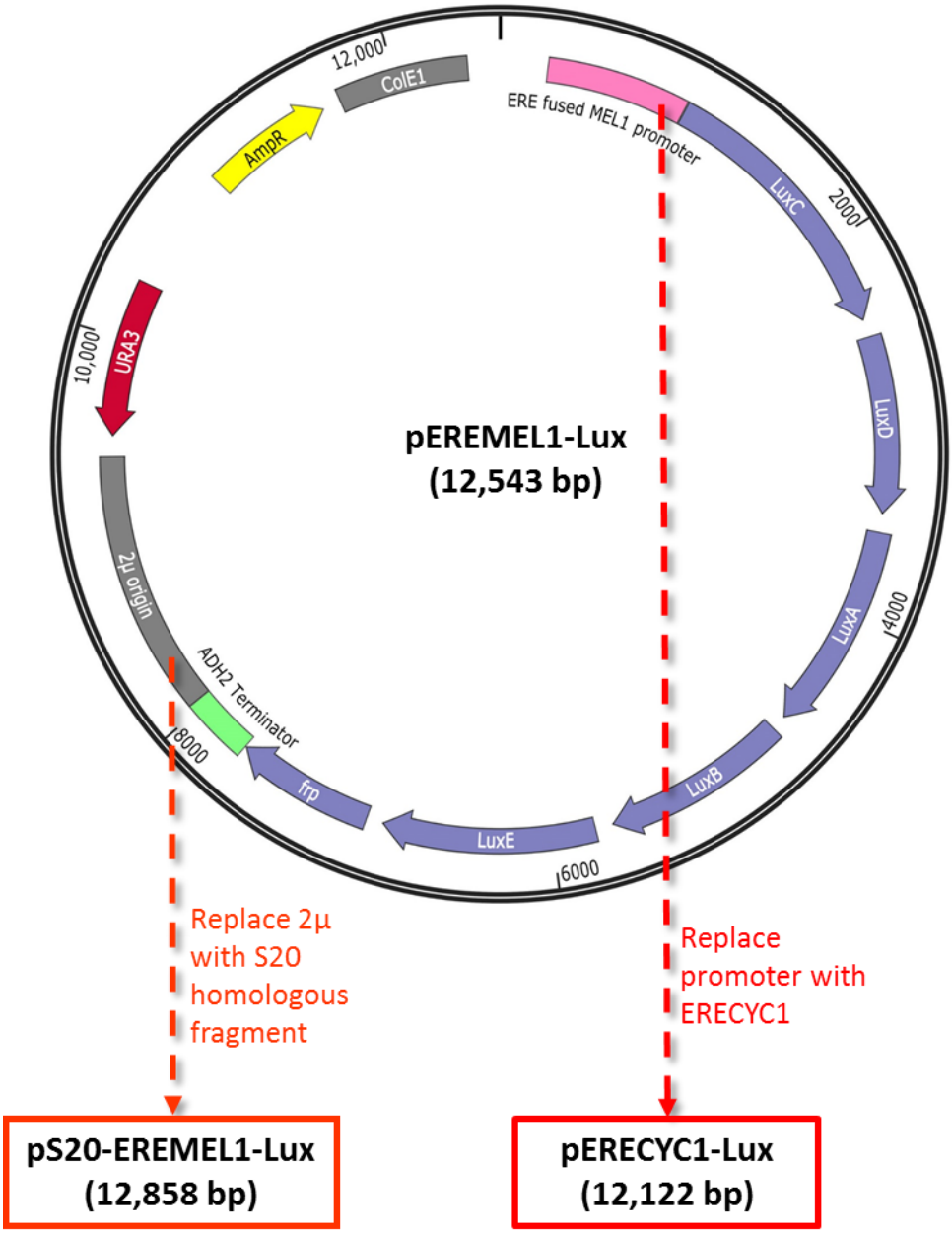


Fig 4.1. Schematic of plasmid pERMEL1-lux, pERECYC1-lux and pS20-EREMEL1-Lux. pERECYC1-lux was constructed by replacing the promoter of pEREMEL1-lux with ERECYC1 promoter. pS20-EREMEL1-lux was constructed by replaced the 2μ origin sequence with the S20 homologous sequence, a DNA fragment amplified from the yeast genome.

hERα gene in the BLYES strain was cloned and designated as hER-y. To clone the hER-y gene, the genomic DNA of *S. cerevisiae* BLYES strain was extracted (YeaStar Genomic DNA Kit, Zymo Research, Irvine, CA) and PCR amplified by Q5 high-fidelity polymerase with ohER_F_BamHI and ohER_R_EcoRI primers. PCR product was gel purified and ligated to p424GPD within BamHI and EcoRI sites. The resulted plasmid was named as p424GPD-hER-y. Cloned hER-y gene was sequenced by ABI 3730 DNA Analyzer at the Molecular Biology Sequencing Facility at University of Tennessee (Thermo Fisher Scientific, Waltham, MA).

Synthesizing wild type hERα gene

Human estrogen receptor alpha was found present in several isoforms, as listed in the database of National Center for Biotechnology Information (NCBI). The 595 amino acids hERα isoform 1 was commonly considered as the full-length wild type form and was chosen for this study and named as hER-wt. The corresponding gene was condon-optimized for yeast expression and synthesized by GenScript (Piscataway, NJ). The synthesized hER-wt gene sequence is presented in Table 4.3.

Construction of hER genome integration plasmids (*pHO-GPDp-hER-CYC1t-KanMX4-HO)*

HO-Poly-KanMX4-HO plasmid was linearized by *Sma*I digestion and gel purified (Wizard SV Gel and PCR Clean-Up System, Promega, Madison, WI). The $GPD_{Promoter}$-polylinker-$CYC1_{Terminator}$ fragment of p424GPD was PCR amplified with HOPoly_cyc1t_GPD_F and HOPoly_cyc1t_GPD_R primers, which contain sequences overlapping the regions flanking the SmaI site of HO-Poly-KanMX4-HO. PCR product was then gel purified and ligated into the linearized HO-Poly-KanMX4-HO plasmid using Gibson Assembly (New England BioLabs, Ipswich, MA)(Gibson, Young et al.

Table 4.3. Synthesized DNA sequences for bioreporter constructions

	Synthesized DNA sequence
Wildtype hERα	ATGACTATGACATTACACACCAAGGCATCCGGTATGGCATTGTTGCACCAAATCCAA GGTAACGAATTAGAACCATTAAATAGACCACAATTGAAAATTCCTTTAGAAAGACC ATTGGGTGAAGTTTATTTGGATTCTTCAAAGCCTGCAGTCTATAATTACCCAGAAGG TGCTGCATACGAGTTTAATGCCGCTGCAGCCGCTAACGCTCAAGTCTATGGTCAAAC CGGTTTGCCTTACGGTCCAGGTTCTGAAGCAGCCGCTTTTGGTTCAAATGGTTTAGG TGGTTTCCCACCTTTGAACTCTGTATCACCTTCCCCATTAATGTTGTTACATCCACCTC CACAATTATCTCCTTTCTTGCAACCACACGGTCAACAAGTTCCTTATTACTTGGAAAA TGAACCATCAGGTTATACTGTCAGAGAAGCAGGTCCTCCAGCCTTTTACAGACCAAA TTCCGATAACAGAAGACAAGGTGGTAGAGAAAGATTAGCTTCTACTAATGACAAAG GTTCTATGGCTATGGAATCAGCAAAGGAAACAAGATATTGTGCCGTATGCAACGAT TACGCTTCTGGTTATCATTACGGTGTTTGGTCATGTGAAGGTTGCAAAGCTTTCTTTA AGAGATCTATCCAAGGTCACAATGATTATATGTGTCCAGCAACCAATCAATGCACTA TTGACAAAAACAGAAGAAAGTCTTGTCAAGCCTGCAGATTGAGAAAGTGTTACGAA GTTGGTATGATGAAAGGTGGTATTAGAAAGGATAGAAGAGGTGGTAGAATGTTGA AACATAAGAGACAAAGAGATGACGGTGAAGGTAGAGGTGAAGTCGGTTCCGCTGG TGACATGAGAGCAGCCAATTTGTGGCCTAGTCCATTGATGATCAAGAGATCTAAGA AAAATTCTTTGGCATTGAGTTTAACCGCCGATCAAATGGTTTCTGCCTTGTTAGACG CTGAACCTCCAATCTTATATTCAGAATACGATCCTACAAGACCATTCAGTGAAGCAT CTATGATGGGTTTGTTAACCAATTTGGCCGACAGAGAATTGGTTCATATGATTAACT GGGCAAAAAGAGTACCAGGTTTTGTTGATTTGACTTTACATGACCAAGTACACTTGT TAGAATGTGCTTGGTTGGAAATCTTAATGATTGGTTTGGTTTGGAGATCTATGGAAC ATCCTGGTAAATTGTTGTTCGCACCAAATTTGTTGTTGGATAGAAACCAGGGTAAAT GTGTCGAGGGTATGGTAGAAATCTTCGACATGTTGTTGGCTACATCCAGTAGATTC AGAATGATGAATTTGCAAGGTGAAGAATTTGTCTGCTTGAAGTCTATAATCTTGTTG AACTCAGGTGTATACACATTTTTGTCTTCAACATTGAAGTCATTGGAAGAAAAGGAT CATATCCACAGAGTTTTGGATAAGATCACTGACACATTGATCCATTTGATGGCTAAG GCAGGTTTGACATTACAACAACAACACCAAAGATTGGCTCAATTGTTGTTGATCTTG TCCCATATCAGACACATGAGTAATAAGGGTATGGAACATTTGTACTCTATGAAGTGT AAGAACGTTGTCCCTTTGTACGATTTGTTGTTGGAAATGTTGGACGCTCATAGATTG CACGCACCAACCTCCAGAGGTGGTGCTAGTGTTGAAGAAACTGATCAATCCCATTT GGCCACAGCTGGTAGTACCTCCAGTCACTCATTACAAAAGTATTACATAACAGGTGA AGCCGAAGGTTTCCCAGCAACAGTCTAATAA
ERECYC1	GAGCTCAAAG**TCAGGTCA**CAG**TGACCTGA**TCAAATCTAGAAGATCCAAAG**TCAGGT** **CA**CAG**TGACCTGA**TCAAACTCGAGCAGATCCGCCAGGCGTGTATATATAGCGTGGA TGGCCAGGCAACTTTAGTGCTGACACATACAGGCATATATATATGTGTGCGACGAC ACATGATCATATGGCATGCATGTGCTCTGTATGTATATAAAACTCTTGTTTTCTTCTT TTCTCTAAATATTCTTTCCTTATACATTAGGACCTTTGCAGCATAAATTACTATACTTC TATAGACACGCAAACACAAATACACACACTAATCTAGA

Bold regions in ERECYC1 are estrogen response element sequences.

2009) following the manufacturer's protocol. Successful construct of pHO-GPDp-CYC1t-KanMX4-HO was verified through PCR and gel electrophoresis.

For yeast genomic integration and expression of hER genes, hER-y and hER-wt were inserted between the $GPD_{promoter}$ and $CYC1_{terminator}$ of pHO-GPDp-CYC1t-KanMX4-HO plasmid respectively (Fig 4.2). Specifically, pHO-GPDp-CYC1t-KanMX4-HO was firstly linearized at the polylinker region by PCR amplification with P424_BamHI_F and P424_EcoRI_R primers. Then primers pairs ohER_gib_HO_F, ohER_gib_HO_R and shER_gib_HO_F, shER_gib_HO_R were used to amplify hER-y and hER-wt respectively. Each primer contains tail sequence overlapping the ends of the linearized pHO-GPDp-CYC1t-KanMX4-HO. PCR amplified hER-y and hER-wt were gel-purified and assembled to linearized pHO-GPDp-CYC1t-KanMX4-HO by Gibson Assembly, producing two integration vectors pHO-GPDp-hERy-CYC1t-KanMX4-HO and pHO-GPDp-hERwt-CYC1t-KanMX4-HO.

Site-directed mutagenesis of wild type human estrogen receptor alpha gene

To obtain mutants of hER-wt gene, site-directed mutagenesis were performed with Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA) following the manufacturer's protocol. The pHO-GPDp-hERwt-CYC1t-KanMX4-HO plasmid was used as the template for hER mutagenesis. Five mutations Asp351Tyr, Glu353Val, Met396Val, Leu525Ala, and Asp538Gly were generated on the integration plasmid respectively. All mutations were verified by sequencing. The oligonucleotides used in the mutagenesis are listed in Table 4.4.

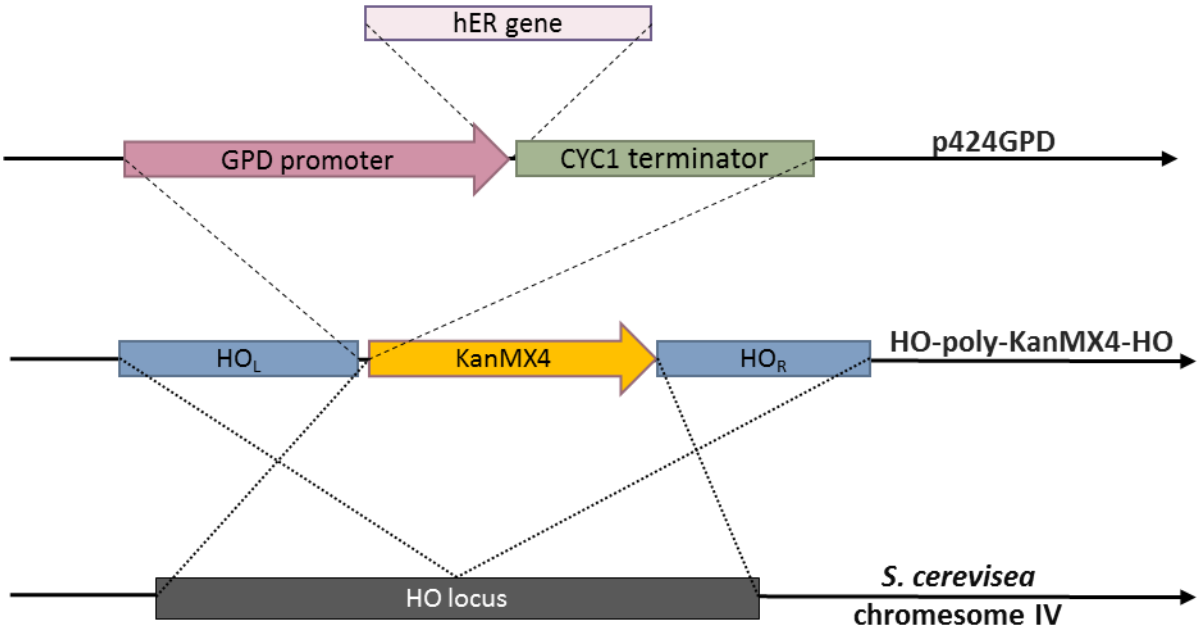


Fig 4.2. Schematic of hER gene expression cassette and genomic integration. hER gene was inserted into p424GPD between the GPD promoter and CYC1 terminator. The whole expression cassette was than inserted into the pHO-poly-KanMX4-HO plasmid. Than the fragment from HO_L to HO_R was PCR amplified and integrated into yeast genome through homologous recombination at the HO locus.

Table 4.4 Oligonucleotides used for human estrogen receptor alpha site-directed mutagenesis

Primer name	sequence
sHER_Asp351Tyr_F	CAATTTGGCCtacAGAGAATTGG
sHER_Asp351Tyr_R	GTTAACAAACCCATCATAGATG
sHER_Glu353Val_F	GCCGACAGAGtATTGGTTCATATG
sHER_Glu353Val_R	CAAATTGGTTAACAAACCCATC
sHER_Met396Val_F	TTGGAGATCTgTGGAACATCC
sHER_Met396Val_R	ACCAAACCAATCATTAAGATTTC
sHER_Leu525Ala_F	TATGGAACATgcgTACTCTATGAAGTG
sHER_Leu525Ala_R	CCCTTATTACTCATGTGTC
sHER_Asp538Gly_F	CCCTTTGTACggtTTGTTGTTGG
sHER_Asp538Gly_R	ACAACGTTCTTACACTTCATAG

Genomic integration and expression of hERα and mutant genes in yeast

The constructed integration plasmid pHO-GPDp-CYC1t-KanMX4-HO carries sequences homologous to the HO locus of *S. cerevisiae* genome, allowing single-copy genomic integration into the same HO locus through homologous recombination(Voth, Richards et al. 2001). hER-y, hER-wt, and hER mutants are all expressed by the same GPD promoter and CYC1 terminator, making strains carrying hER variants easily comparable.

To perform the integration, pHO-GPDp-CYC1t-KanMX4-HO carrying either hER-y, hER-wt or hER mutants were PCR amplified from HO_L to HO_R region with HO_F and HO_R primers. PCR products were then directly transformed into *S. cerevisiae* BY4742 following the LiAc/ssDNA/PEG method previously described(Gietz and Schiestl 2007). Transformants were selected on the aforementioned YPD agar plates with G418 antibiotic selection. Genomic integration was verified by genomic DNA PCR amplification and gel electrophoresis. Expression of hER was further verified by reverse transcriptase PCR.

Construction of integration plasmid *pS20-EREMEL1-Lux*

pEREMEL1-Lux is a self-replicative plasmid contains estrogen response element (ERE) fused MEL1 promoter, which can trigger the expression of downstream light-producing luxCDABE genes when bounded by activated estrogen receptor proteins.

To allow genomic integration of this bioluminescent reporter plasmid, the 2μ element responsible for plasmid self-replication within the yeast host was replaced by a 1.6kb DNA amplified from yeast genome (Fig 4.1), which not only disrupted the self-replicative feature of the plasmid, but also provided homologous sequence, making genomic integration feasible. The

locus of the 1.6 kb DNA was located at Chromosome XVI and was chosen based a previous study characterizing efficient chromosomal integration sites for heterologous gene expression in yeast(Dongmei Bai, Verena et al. 2009). For convenience purpose, the 1.6 kb DNA was named as S20. To replace the 2µ with S20, PCR was performed on pEREMEL1-Lux using S20_pERE_F and S20_pERE_R primers, which produced linearized pEREMEL1-lux without the 2micron element, and also added overlapping sequence to the S20. S20 was PCR amplified from yeast genomic DNA with 20_up_F and 20_down_R primers and then assembled to the linearized plasmid by gibson assembly, generating pS20-EREMEL1-Lux.

Construction of bioluminescence reporter vector *pERECYC1-lux*

pERECYC1-Lux was generated by replacing the promoter EREMEL1 in the pEREMEL1-Lux plasmid with the ERECYC1 sequence (Fig 4.1). ERECYC1 sequence was an estrogen response element fused CYC1 promoter successfully used in a previously developed yeast estrogen bioassay(Toine, Richard et al. 2004). The ERECYC1 DNA was synthesized by Genscript (Piscataway, NJ), and the sequence is presented in Table 4.3.

pEREMEL1-Lux was linearized by PCR amplification with pERE_Up_pro and pERE_Down_pro primers, which excluded the EREMEL1 region. Synthesized ERECYC1 DNA was then PCR amplified with ERECYC1_F+pERE and ERECYC1_R+pERE primers which also added tail sequences overlapping the linearized pEREMEL1 plasmid. ERECYC1 was then assembled to the linearized plasmid by gibson assembly, generating pERECYC1-Lux. Successful ligation was verified by sequencing.

Transformation and construction of yeast estrogen bioluminescent reporters

Yeast strains carrying hER-y and hER-wt were transformed with pERECYC1-lux, pEREMEL1-lux and pS20-EREMEL1-lux respectively following the previously described method (Gietz and Schiestl 2007). Prior to yeast transformation, pS20-EREMEL1-lux was linearized at the middle of the S20 region by PCR with 20-up_R and 20-down_F primers, allowing genomic integration at the S20 site of yeast chromosome XVI. Yeast strains carrying hER mutants were transformed with pERECYC1-lux only. Transformed yeast colonies were selected on YSD(-ura) agar plate and then maintained on YSD (-ura, G418) agar plate. All strains constructed are listed in Table 4.5.

Bioreporter strain performance evaluation

To test the constructed strains' response time to estrogen and quantitative luminescence, 10 µL of 10E-6 M 17β-estradiol in methanol were pipetted to 96 well plate and air-dried. Then a fresh colony of each strain was picked and diluted into YSD (-ura) liquid medium to reach OD_{600} ~0.5. Then 100 µL of the diluted culture were added to each well of the 96-well plate. Negative controls (yeast culture without estrogen added) were included for each strain and the exposure were conducted in triplicate. To evaluate the EC_{50} of 17β-estradiol for each strain, each strain was grown overnight at 28°C, 200 rpm from fresh colony until OD_{600} reached ~0.8. Before the test, 10 uL of serial diluted 17β-estradiol in methanol were added into the 96-well plate and allowed air-dried. Then 100 uL of yeast culture was added into each well. Bioluminescence was measured by BioTek Synergy2 Multilabel reader, with default gain setting of 135, integration time of 1 s/well. Experiment was performed in triplicate. Solvent blanks (cell+medium+solvent) and true blanks (cell+medium) were included as negative controls.

Table 4.5 Constructed *Saccharomyces cerevisiae* bioluminescent estrogen reporters

Strain Name	Form of reporter plasmid	Form of hERα	hERα mutations
ERy-pMEL	pEREMEL1-lux	hER-y	Lys362Glu, Asn413Asp, extra 20 AA residues
ERy-pCYC	pERECYC1-lux	hER-y	
ERy-inteMEL	pS20-EREMEL1-lux (integrated)	hER-y	
ERwt-pMEL	pEREMEL1-lux	hER-wt	Wild type
ERwt-pCYC	pERCYC1-lux	hER-wt	Wild type
ERwt-inteMEL	pS20-EREMEL1-lux (integrated)	hER-wt	Wild type
ER351-pCYC	pERECYC1-lux	hER-351	Asp351Tyr
ER353-pCYC	pERECYC1-lux	hER-353	Glu353Val
ER396-pCYC	pERECYC1-lux	hER-396	Met396Val
ER525-pCYC	pERECYC1-lux	hER-525	Leu525Ala
ER538-pCYC	pERECYC1-lux	hER-538	Asp538Gly

hER-wt: wildtype 595 amino acids human estrogen alpha
hER-y: human estrogen receptor alpha in *S. cerevisiae* BLYES strain(Sanseverino, Gupta et al. 2005).

Data analysis

Chemical exposure response was considered significant when the bioluminescence increase was higher than three times the background bioluminescence standard deviation (3-sigma rule, $p<0.003$). To calculate the EC_{50} of 17β-estradiol for each strain, bioluminescence reading were plotted against the log concentration, generating a sigmoidal curve. EC50 of each test was calculated using the 4 variables sigmoidal curve fitting function in GraghPad Prism 5.0 (GraphPad Software, La Jolla, California).

4.2.2 Results and Discussions

Response time and dynamic range of hER-y and hER-wt bioreporter strains

A goal of this study was to create comparable bioreporters strains expressing hER mutant genes. Therefore it was necessary to firstly establish proper constructs that deliver stable and comparable performance in estrogen detection. The original BLYES previously developed have been extensively used in many applications including potential estrogenic compound screening, creek water, wastewater, sludge, soil testing, etc(Wang, Eldridge et al. 2015). However the genetic background of the strain wasn't informed in the literature. Therefore *S. cerevisiae* BY4742 strain was chosen as the new host for bioreporter construction. The hER gene chromosomally integrated in the BLYES strain (designated as hER-y) was cloned and initially used as the wild type for new bioreporter construction. Nevertheless, sequencing revealed that hER-y contains two missense mutations (Lys362Glu, Asn413Asp), as well as an extra 20 amino acids fragments at the AF-1 domain that wasn't reported in previous literature and database yet (Fig 4.3). Therefore a true wild type, the 595 amino acids hER-wt gene, was synthesized and used in this study.

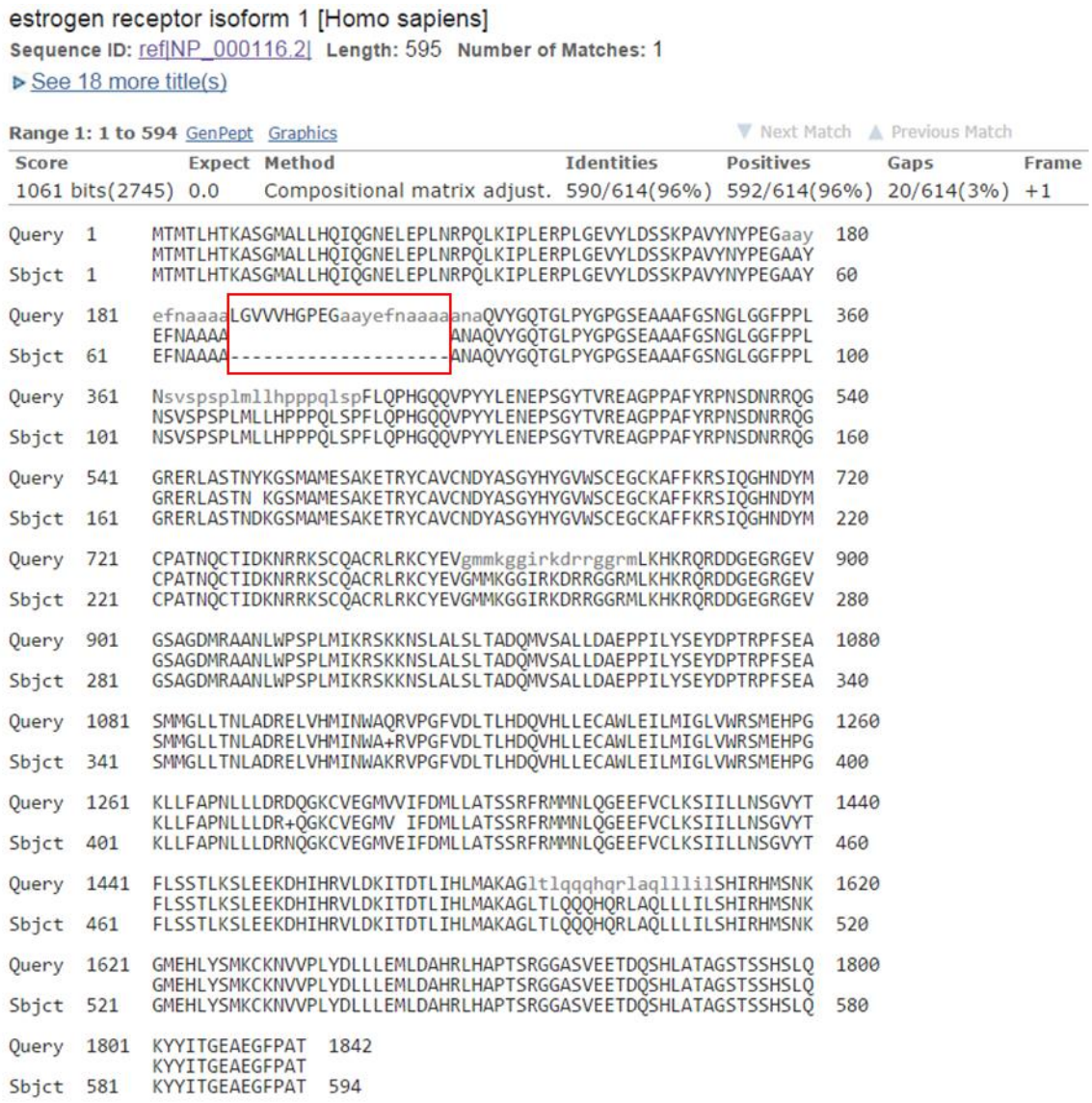


Fig 4.3. Translated hER-y sequence aligned with wildtype estrogen receptor sequence. The hER-y protein contain extra 20 amino acids (marked in red box), as well two missense mutations Lys362Glu, Asn413Asp.

Two plasmids sharing the same lux genes except the ERE fused promoter, pEREMEL1-lux and pERECYC1-lux, were evaluated for their bioluminescence production with hER-y and hER-wt respectively. Besides, single-copy genomic integration of pS20-EREMEL1-lux was also tested for bioluminescence production performance. A total of six strains were generated based on the *S. cerevisiae* BY4742 strain, as listed in Table 4.5.

To determine the performance of hER-wt and hER-y bioreporter strains, natural estrogen 17β-estradiol was used as the standard chemical to verify proper ligand-receptor interaction and transcriptional activation, as well as to evaluate the response time and the dynamic range of bioluminescence output. All 6 strains were exposed to 1E-7 M of 17β-estradiol, a saturation concentration at which estrogen bioreporters produce the maximum response according to previous studies (Toine, Richard et al. 2004, Sanseverino, Eldridge et al. 2009). Bioluminescence monitored throughout 24 hours was plotted in Fig. 4.4. All six strains showed no background bioluminescence when no estrogen was present; the background bioluminescence was indistinguishable from the background reading of the blanks. Significant bioluminescence response was detected after 1 hour of estrogen exposure for ERwt-pMEL, ERy-pMEL, ERy-pCYC strains, though the signals were low. After two hours, ERwt-pCYC and ERy-inteMEL strains produced significant bioluminescence as well. ERwt-inteMEL strain failed to produce detectable bioluminescence throughout the whole exposure experiment, while ERy-inteMEL strain produced detectable yet very weak bioluminescence response with the maximum output averaging 146.3 counts/s. hER-y based strains ERy-pMEL and ERy-pCYC produced the maximum bioluminescence at the 12^{th} hour, with averages of 43453.2 and 25999.8 counts/s respectively. In comparison, hER-wt based ERwt-pCYC produced maximum bioluminescence at the 11th hour

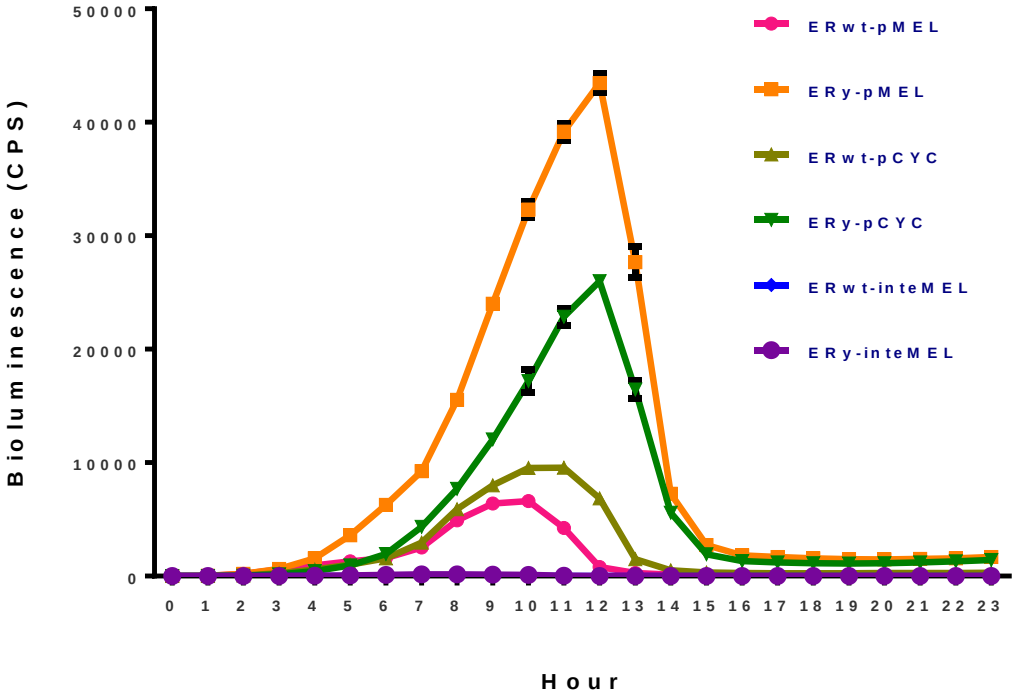


Fig 4.4. Time course response of six estrogen bioreporters to 1E-7 M of 17β-estradiol. (Mean and standard deviation, n=3)

with an average of 9567.5 counts/s; ERwt-pMEL strain produced maximum response at the 10th hour with an average of 6615 counts/s. These results together indicate these strains have similar response time to 17β-estradiol, yet the dynamic range of light output differs considerably.

When ERy-pMEL and ERwt-pMEL strain were initially constructed, hER-y based ERy-pMEL produced significantly higher maximum bioluminescence than hER-wt based ERwt-pMEL strain. To determine whether this difference was the result of the sequence difference between hER-y and hER-wt, the promoter of pEREMEl1-lux was replaced by a previously developed ERE fused CYC1 promoter(Toine, Richard et al. 2004), generating the pERECYC1-lux plasmid. As demonstrated in Fig 4.4, ERy-pCYC1 strain still produced much higher maximum bioluminescence upon exposure to 17β-estradiol compared to the ERwt-pCYC1 strain. Additionally, for the two strains with integrated pS20-EREMEL1-lux (ERy-inteMEL and ERwt-inteMEL), ERy based strain was able to produce detectable light output while the ERwt based strain could not. These findings together confirmed that hER-y gene was responsible for the higher bioluminescence production. Sequencing of hER-y revealed that hER-y gene contained two missense mutations (Lys362Glu, Asn413Asp), as well as an extra 20 amino acid residues fragment in the DNA binding domain (Fig 4.3). BLAST in NCBI database did not demonstrate any previous record regarding this extra fragment. Whether the elevated bioluminescence production was the result of the mutations or the extra fragment requires further investigation.

The purpose of constructing ERwt-inteMEL and ERy-inteMEL strains with chromosomally integrated bioluminescence plasmid was to allow all the bioreporters having identical number (single copy) of bioluminescence production cassettes, which can minimize the variables when comparing strains carrying different hER mutations. However the potential risk of such

construction is that low copy number of bioluminescence genes in the yeast cell may not be able to produce sufficient level of signal. This may explain why in this study, ERwt-inteMEL strain failed to produce detectable bioluminescence. Although hER-y based strain ERy-inteMEL produced detectable signal with the integrated pEREMEl1-lux, the light output was also too weak to be applicable for the downstream mutagenesis comparison study.

Half-maximal effective concentration (EC_{50}) of hER-y and hER-wt based estrogen bioreporters

EC50 is the primary metric used to quantify and compare the potency of estrogenic compounds to activate with estrogen receptor. Therefore to determine which bioreporter construct to be used for the downstream mutagenesis study, EC50 of all six strains in exposure to 17β-estradiol were compared. Overnight culture (in 2% glucose YSD -ura medium) of the six hER-y and hER-wt based strains were exposed to serially diluted 17β-estradiol and bioluminescence was monitored every hour. Except for the two strains that have pEREMEL1-lux integrated in the genome (ERy-inteMEL and ERwt-inteMEL), all other four strains produced detectable bioluminescence and a peak response at 6 hours then decreased. Therefore the 6th hour bioluminescence was plotted against the log concentration of 17β-estradiol (Fig 4.5). All four strains produced sigmoidal exposure curves at this time point. Similar to the previous test, ERy-pMEL demonstrated higher dynamic range, with maximum light output reaching ~8000 counts/s. ERwt-pCYC and ERy-pCYC showed slightly smaller dynamic range, with maximum bioluminescence peaking at averages of 5186 and 4613 counts/s respectively. ERwt-pMEL, produced a weaker response in this test, with maximum bioluminescence averaging 2085 counts/s. The EC50 of 17β-estradiol for each of the four strains were calculated and listed in Table 4.6. The EC50 of ERwt-pMEL, ERwt-pCYC and

Table 4.6. Half-maximal effective concentration (EC_{50}) of 17β-estradiol for each strain through curve fitting

	ERwt-pMEL	ERy-pMEL	ERwt-pCYC	ERy-pCYC	ERwt-inteMEL	ERy-inteMEL
EC_{50} (M)	5.86E-09	9.35E-10	3.71E-09	1.16E-09	-	-
R^2 (curve fitting)	0.97	1.00	0.98	0.99	-	-

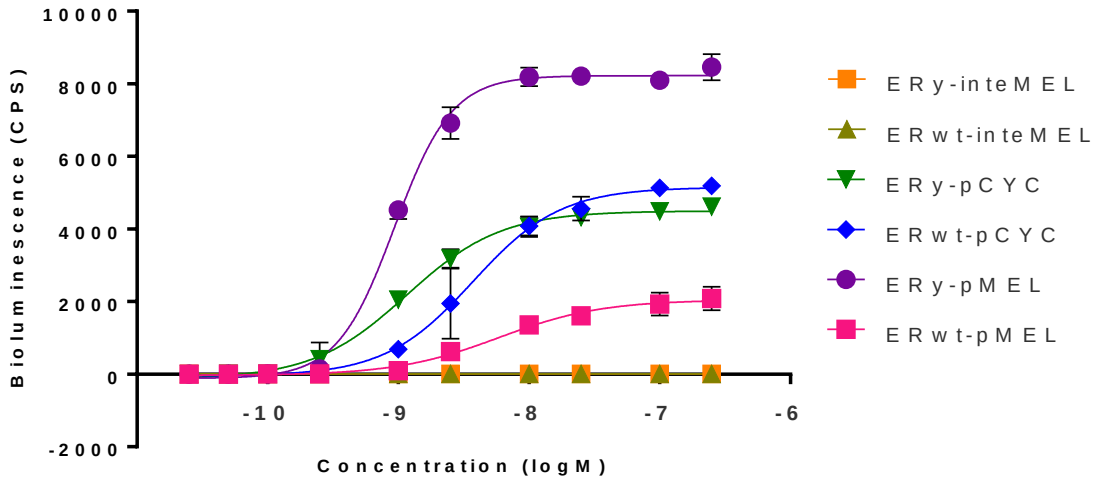


Fig 4.5. Dose response curve of 6th hour 17β-estradiol exposure for overnight cultured strains.

Values are Mean ±SD, n =3.

ERy-pCYC were all in the 10E-9 molar range, while the ERy-pMEL showed slightly lower EC50 of 10E-10 range.

For downstream hER mutagenesis study, ERwt-pCYC was chosen as the wildtype base strain for its maximum bioluminescence response and EC50 in exposure to 17β-estradiol. ERwt-pCYC was also favored as the ERE fused CYC1 promoter was successfully used in yeast GFP based estrogen bioreporters in previous studies (Bovee, Helsdingen et al. 2004, Toine, Richard et al. 2004).

Optimization of ERwt-pCYC strain based chemical bioassay

One phenomenon that was found for all the constructed strains including the ERwt-pCYC was that for overnight cultures grown with 2% glucose YSD medium, the bioluminescence of 17β-estradiol exposure quickly reached the maximum at 6 hours and then drastically declined. The bioluminescence output of these strains was much shorter compared to *S. cerevisiae* BLYES, whose maximum bioluminescence lasts at least 12 hours(Sanseverino, Gupta et al. 2005). Since ERwt-pCYC was chosen for the new estrogen reporter base construct, it was necessary to extend the response time to optimize the chemical exposure bioassay due to the assay format and utility in data analysis. Considering that GPD promoter and CYC1 terminator was used for hER expression, a further literature search revealed a previous finding that the performance of yeast GPD promoter can be significantly suppressed by limiting glucose concentration in the culture(Siavash, Verena et al. 2010). To test whether glucose content was causing the altered bioluminescence response, an overnight culture of ERwt-pCYC with OD 1.64 was 1:2 diluted by YSD (-ura) media with 2%, 4% and 6% (w/v) of glucose. Diluted culture was then exposed to serial diluted 17β-estradiol and bioluminescence reading was recorded at each hour for 24 hours. The

maximum bioluminescence of the diluted ERwt-pCYC culture was clearly enhanced by the presence of added fresh medium (Fig 4.6). Compared to non-diluted culture, all diluted cultures showed significantly higher bioluminescence response. When comparing the cultures diluted with different glucose concentration, 2% glucose YSD diluted culture reached peak bioluminescence at 11 hours, while 4% glucose YSD diluted culture maintained bioluminescence to 18 hours. The culture diluted with 6% glucose YSD maintained the increase of bioluminescence till 24 hours, however its maximal light output was lower compared to the 4% glucose YSD diluted culture from 7 to 18 hours.

The EC50s of 17β-estradiol for the original and diluted cultures were also calculated for each hour once a full sigmoidal response curve was present (Fig 4.7). The EC50 of all diluted culture were within the 10E-9 M range. 2% and 4% glucose YSD diluted cultures demonstrated similarly stable EC50 throughout the monitoring period, with the EC50 gradually shift towards the lower 10E-9M range. Based on the test results, 4% glucose YSD were chosen as the media for yeast bioassay with 1:2 dilution before chemical exposure.

To test whether this modified protocol can yield EC50, 17β-estradiol standard exposure tests were repeated six times using different batch of ERwt-pCYC culture (Fig 4.8). The length of exposure varied from 12 to 15 hours in these tests, and the EC50 ranged from 0.96E-9 M to 5.79E-9 M, with an average of 2.44E-9 M and standard deviation of 1.95E-9 M, suggesting that the ERwt-pCYC strain and the modified protocol is capable of evaluating estrogenic compounds reliably and reproducibly.

Compared to the previously constructed *lux*-based bioreporter *S. cerevisiae* BLYES, which was reported with EC50 of (2.4±1.0)E-10 M for 17β-estradiol, ERwt-pCYC strain demonstrated lower

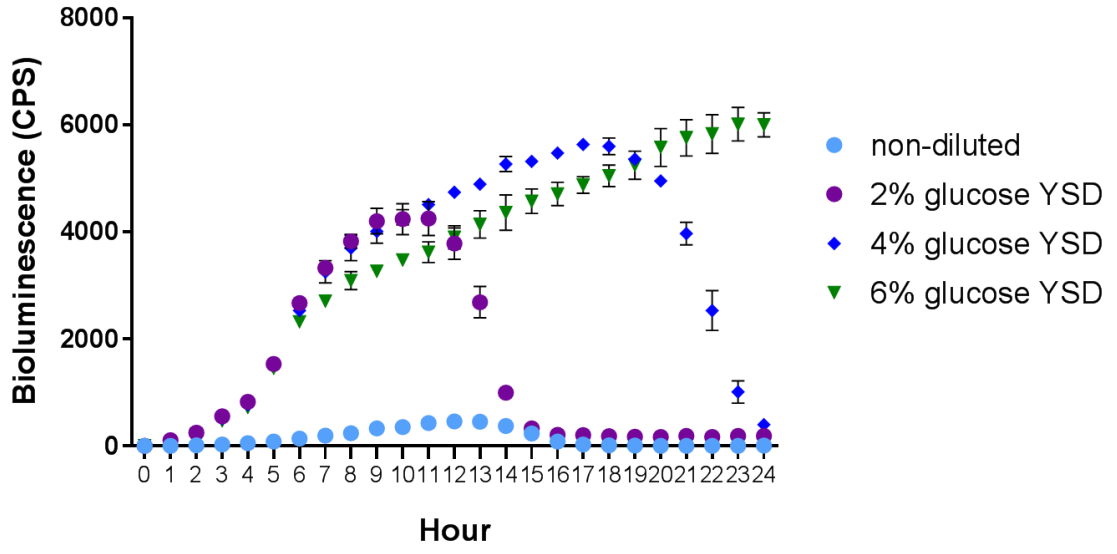


Fig 4.6. Maximum bioluminescence of 1:2 diluted ERwt-pCYC overnight culture in YSD medium with different glucose concentration.

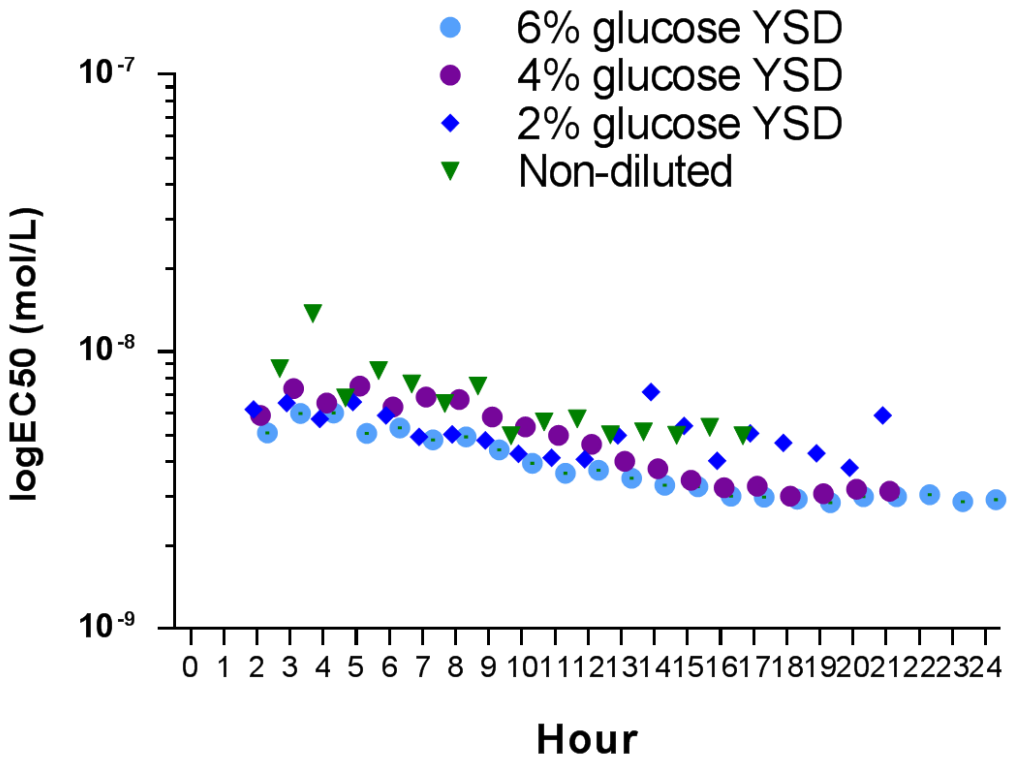


Fig 4.7. EC50 of 1:2 diluted ERwt-pCYC overnight culture in YSD medium with different glucose concentration.

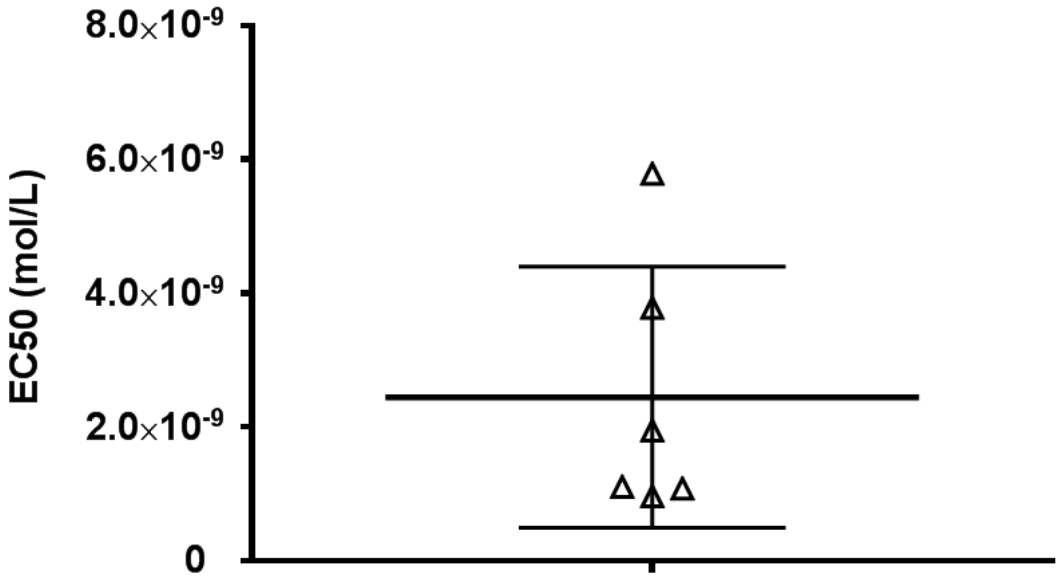


Fig 4.8. Range of EC50 of 17β-estradiol responded by ERwt-pCYC strain. Triangles are each data point. Bars are the mean and standard deviation.

sensitivity, with average EC50 of 17β-estradiol about one magnitude less than that of BLYES(Sanseverino, Gupta et al. 2005). Considering the molecular constructs of ERwt-pCYC and BLYES strain are different on several aspects, i.e. the design/layout of bioluminescent plasmid, estrogen receptor and its expression cassette etc., the potential cause of ERwt-pCYC's reduced sensitivity requires further investigations. Yet the performance of the ERwt-pCYC strain was adequate for the purpose of this study, for the strain demonstrated zero background bioluminescence, good reproducibility with reliable level of sensitivity.

4.3. Evaluating hERα Mutants' Response to Putative Estrogenic EDCs.

With ERwt-pCYC strain selected as the wild type hERα bioreporter, 5 mutant strains, each carrying a missense mutation at hERα ligand binding domain and the same pERECYC1-lux plasmid, were used to evaluate how these mutations alter the response of estrogen receptor to different EDCs. A total of 12 estrogenic compounds, including natural estrogen, synthetic estrogens, phytoestrogens, and industrial chemicals were evaluated by these strains.

4.3.1 Materials and Methods

Chemical dilutions

All tested estrogenic compounds including the structure and CAS registry number are listed in Table 4.1. To perform the chemical exposure bioassay, E2, DES, 4-nonylphenol, bisphenol-A, tamoxifen, EE2, mestranol, resveratrol, *S*-Equol were dissolved in distilled methanol. Genistein, daidzein and coumestrol were dissolved in dimethyl sulfoxide (DMSO). All chemicals were initially prepared at 1E-2 M concentration, and diluted 1:2 for twice, yielding the 5E-3 M and 2.5E-3M

dilution. Then each of the three dilutions were 1:10 serial diluted for 8 times, making the final dilutions ranging from 1E-2 to 2.5E-11 M.

Strain, culture condition

Six genetically engineered *S. cerevisiae* bioluminescent reporter strains, ERwt-pCYC, ER351-pCYC, ER353-pCYC, ER396-pCYC, ER525-pCYC and ER538-pCYC were used in this comparison study (Table 4.5). On the day before chemical test, each culture was started from a fresh colony in 5 mL of 4% glucose YSD (-ura) liquid medium and grown overnight at 28 °C, 200rpm until reaching OD 1.0. The culture was then 1:2 diluted in 4% glucose YSD (-ura) medium prior to chemical exposure.

Chemical exposure bioassay

For chemicals soluble in methanol, 10 µL of each serial dilution were placed in each well of the white 96 well plate and air-dried. For chemicals dissolved in DMSO, 1 µL of each serial dilutions were added into each well of the white 96 well plate. Then 100uL of 1:2 diluted yeast culture was added to each well to initiate the chemical exposure. For each test, solvent blanks (cell+medium+solvent) and true blanks (cell+medium) were included as negative controls. Each chemical were tested by all the six yeast estrogen bioreporters on the same day. Chemical bioassays were performed in three biological replicates, and each with two technical replicates. Loaded 96-well plates were covered with sealing films and incubated at 28 °C for 15 hours. Bioluminescence was measured by BioTek Synergy2 Multilabel reader, with default gain setting of 135, integration time of 1 s/well.

Data analysis

Chemical exposure response was considered significant when the bioluminescence increase is higher than three times of the background bioluminescence standard deviation (3-sigma rule, $p<0.003$). To calculate the EC_{50} of each chemical for each strain, bioluminescence readings were plotted against log concentration. EC_{50} of each chemical for each biological replicate was calculated using four-parameter dose-response curve fitting in GraphPad Prism 5.0 (GraphPad Software, La Jolla, California). To determine the significance of difference between the chemical responses of wild-type strain and mutant strain to each ligand, Student's t-test was performed using log EC50 values from each test.

Two way hierarchical clustering was performed using ClustVis(Metsalu and Vilo 2015). Average EC50 values from the yeast assay were –log transformed and missing values were filled with 0.01 prior to the clustering. For hierarchical clustering, rows were centered; unit variance scaling was applied to rows. Both rows and columns were clustered using correlation distance and Ward linkage.

4.3.2 Results and Discussion

To determine whether hER mutations have altered responses to different estrogenic EDCs, yeast based estrogen receptor bioreporter assay was selected as the platform to evaluate transcriptional activation triggered by ligand activated estrogen receptor. With ERwt-pCYC strain selected as the wild type hERα bioreporter, 5 mutant strains, each carrying a missense mutation at hERα ligand binding domain and the same pERECYC1-lux plasmid, were used to evaluate how these mutations modulate the response of estrogen receptor to different EDCs. A total of 12

estrogenic compounds, including natural estrogen, synthetic estrogens, phytoestrogens, and industrial material were evaluated by these strains. EC50 of all tested chemicals for each hER yeast reporter are presented in Table 4.7 and Fig 4.9. The dose-response curves for all tested chemicals are presented in Fig 4.10.

Response of wildtype bioreporter ERwt-pCYC to 12 estrogenic EDCs

The wildtype bioreporter produced quantifiable response to all the tested estrogenic compounds except for daidzein (detectable yet non-reportable response was observed) (Fig 4.11, Table 4.7). Among these estrogenic compounds, natural estrogen E2 and synthetic estrogen EE2 triggered the strongest response, with EC50 of (1.51±0.32)E-09 M and (9.68±1.06)E-10 M respectively, the lowest among all the tested chemicals. Synthetic hormones DES and mestranol, and the phytoestrogen coumestrol also triggered lower and moderately strong responses, with EC50 ranging from (8.67±1.99)E-09, (3.93±1.28)E-08 to (5.78±0.84)E-08 M respectively. Although daidzein exposure produced weak non-reportable response, its biotransformed product S-equol triggered significantly higher response with EC50 of (1.10±0.36)E-06 M. Another phytoestrogen genistein showed similar level of EC50 of (1.45±0.10)E-06 M. The two widely used industrial compounds nonylphenol and BPA produced relatively weak response with EC50 of (3.87±0.79)E-06 and (1.05±0.21)E-05 M respectively. The selective estrogen receptor modulator tamoxifen exhibited weak estrogenic response as well, yielding EC50 of (1.36±0.12)E-05 M.

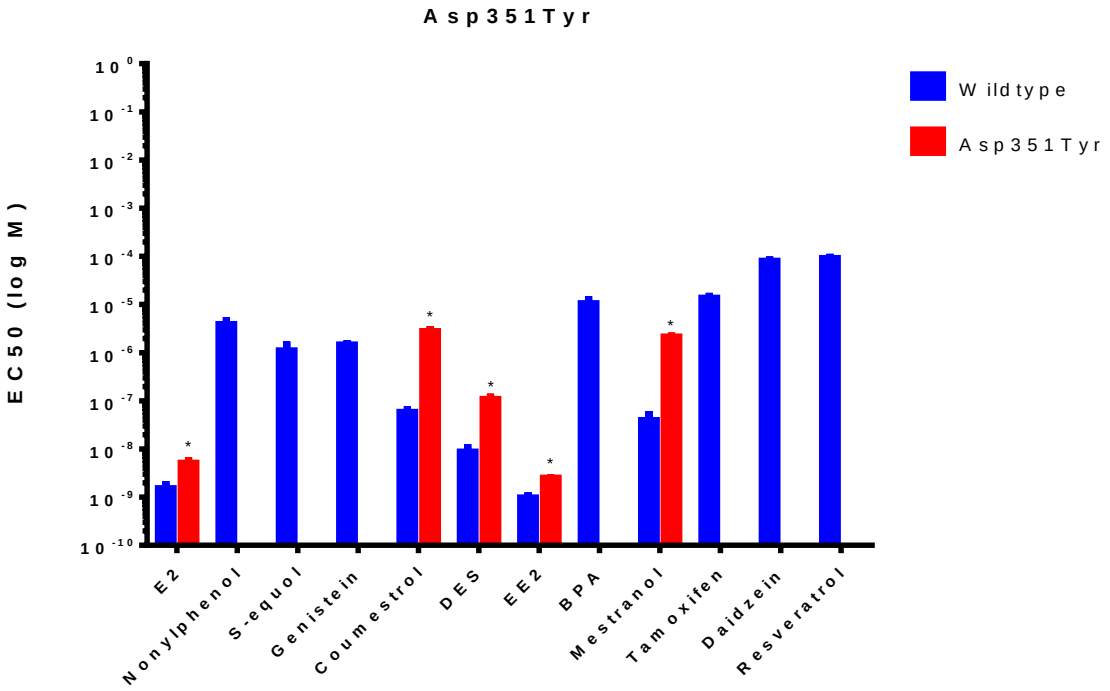
Table 4.7. Half-maximal effective concentration (EC50) of 12 estrogenic compounds evaluated by wild type and mutant hERα yeast estrogen bioreporters

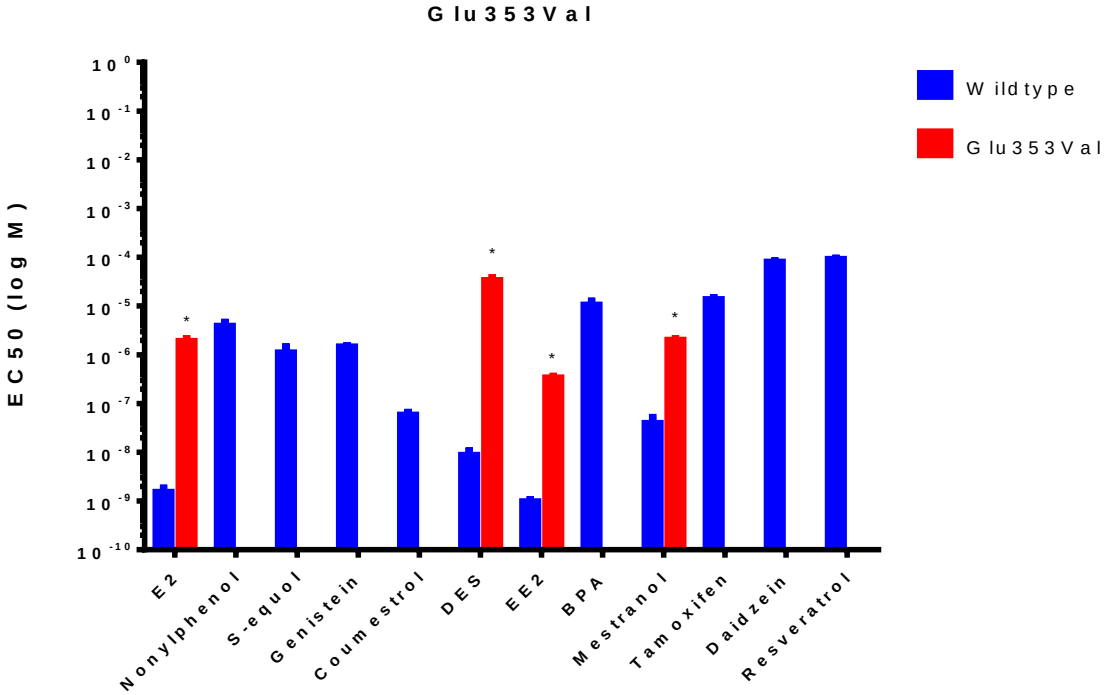
	Wildtype (mol/L)	**Asp351Tyr (mol/L)**	**Glu353Val (mol/L)**	**Met396Val (mol/L)**	**Leu525Ala (mol/L)**	**Asp538Gly (mol/L)**
E2	(1.51±0.32) E-09	(5.09±0.60) E-09*	(1.90±0.26) E-06*	(1.69±0.81) E-09	(3.82±1.03) E-08*	(1.14±0.04) E-09
EE2	(9.68±1.06) E-10	(2.49±0.03) E-09*	(3.36±0.27) E-07*	(7.11±0.18) E-10	(2.12±0.46) E-08*	(4.01±0.74) E-09*
DES	(8.67±1.99) E-09	(1.08±0.12) E-07*	(3.36±0.45) E-05*	(8.09±2.12) E-09	(2.36±0.51) E-07*	(1.24±0.16) E-08*
Mestranol	(3.93±1.28) E-08	(2.12±0.10) E-06*	(1.99±0.16) E-06*	(2.39±0.47) E-08	(3.07±0.36) E-05*	(9.21±1.69) E-09*
Tamoxifen	(1.36±0.12) E-05	-	-	(1.08±0.11) E-05	n.r	(2.50±0.17) E-05*
Daidzein	n.r	-	-	n.r	-	(1.13±0.06) E-05*
S-equol	(1.10±0.36) E-06	-	-	(1.39±0.63) E-06	-	(1.18±0.08) E-07*
Genistein	(1.45±0.10) E-06	n.r	-	(1.21±0.25) E-06	n.r	(2.23±0.06) E-07*
Coumestrol	(5.78±0.84) E-08	(2.77±0.23) E-06*	-	(6.27±0.38) E-08	-	(3.76±0.34) E-08*
Resveratrol	(9.14±0.45) E-05	-	-	(7.85±0.74) E-05	-	(1.26±0.17) E-04*
Nonylphenol	(3.87±0.79) E-06	-	-	(3.78±0.07) E-06	(3.88±0.22) E-05*	(1.70±0.03) E-07*
BPA	(1.05±0.21) E-05	n.r	-	(8.27±1.22) E-06	(1.28±0.12)-04*	(3.52±0.62) E-06*

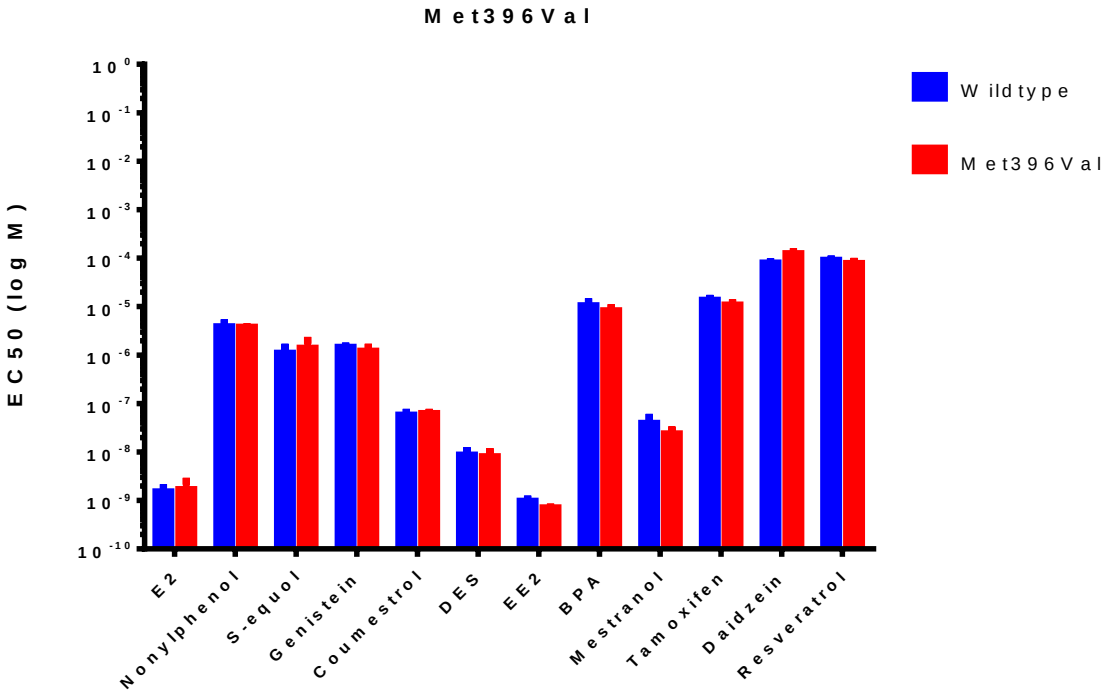
Results are expressed as mol/L concentration (mean ± SD for biological triplicates). Results indicated with * are mutant responses that are significantly different from the wild type response (Student's t-test, using –log values, $p<0.05$). "n.r": detectable response but EC50 non-reportable. "-": no detectable response.

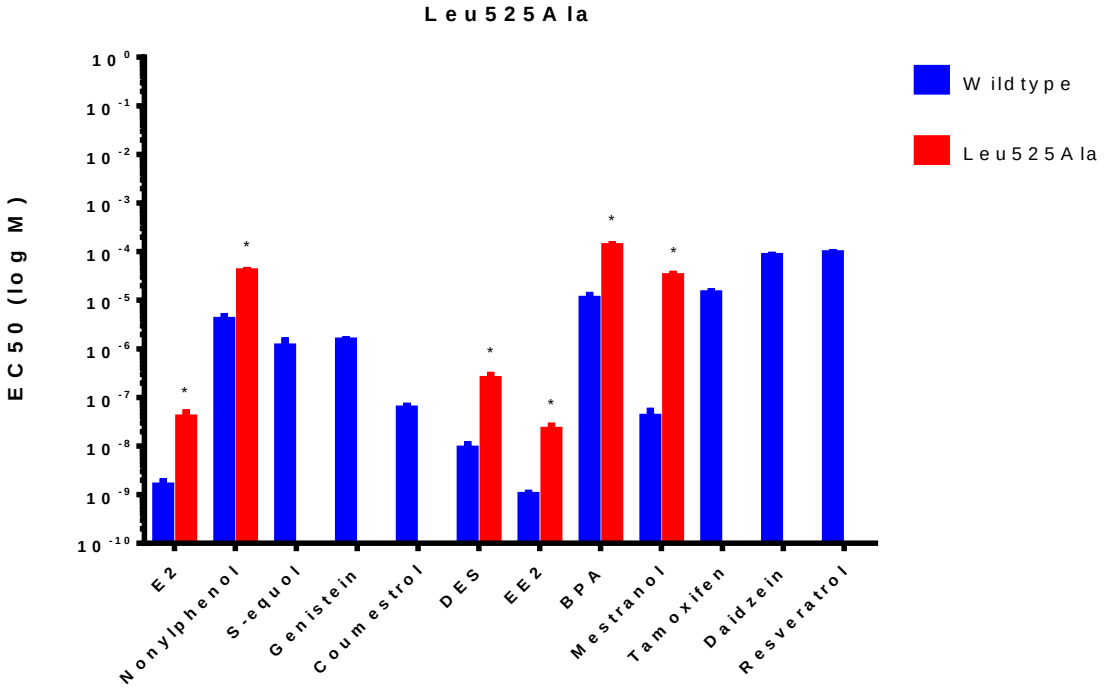
Fig 4.9. EC50 of 12 ligands evaluated by wildtype and mutant hERα yeast estrogen bioreporters.

(Values presented as mean ± SD obtained from biological triplicate. *: $p<0.05$)


Asp351Tyr
EC50 (log M)
10 0
10 -1
10 -2
10 -3
10 -4
10 -5
10 -6
10 -7
10 -8
10 -9
10 -10
Wildtype
Asp351Tyr
*
E2
Nonylphenol
S-equol
Genistein
Coumestrol
DES
EE2
BPA
Mestranol
Tamoxifen
Daidzein
Resveratrol


Glu353Val
EC50 (log M)
10 0
10 -1
10 -2
10 -3
10 -4
10 -5
10 -6
10 -7
10 -8
10 -9
10 -10
Wildtype
Glu353Val
*
E2
Nonylphenol
S-equol
Genistein
Coumestrol
DES
EE2
BPA
Mestranol
Tamoxifen
Daidzein
Resveratrol


Met396Val
EC50 (log M)
10 0
10 -1
10 -2
10 -3
10 -4
10 -5
10 -6
10 -7
10 -8
10 -9
10 -10
Wildtype
Met396Val
E2
Nonylphenol
S-equol
Genistein
Coumestrol
DES
EE2
BPA
Mestranol
Tamoxifen
Daidzein
Resveratrol


Leu525Ala
EC50 (log M)
10 0
10 -1
10 -2
10 -3
10 -4
10 -5
10 -6
10 -7
10 -8
10 -9
10 -10
Wildtype
Leu525Ala
*
E2
Nonylphenol
S-equol
Genistein
Coumestrol
DES
EE2
BPA
Mestranol
Tamoxifen
Daidzein
Resveratrol

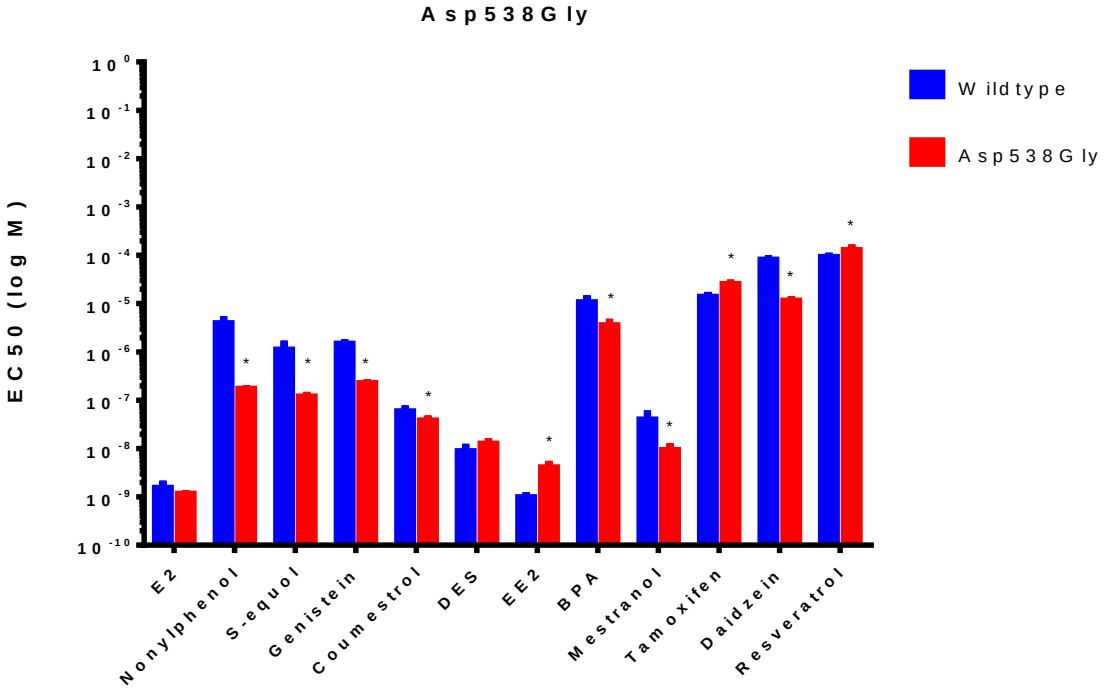
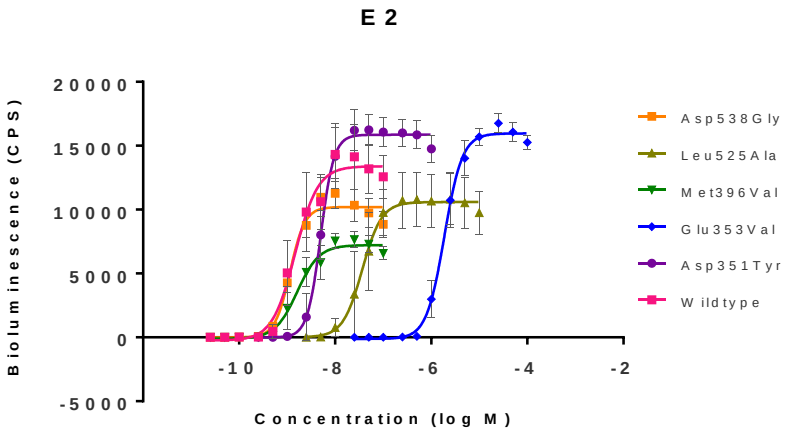
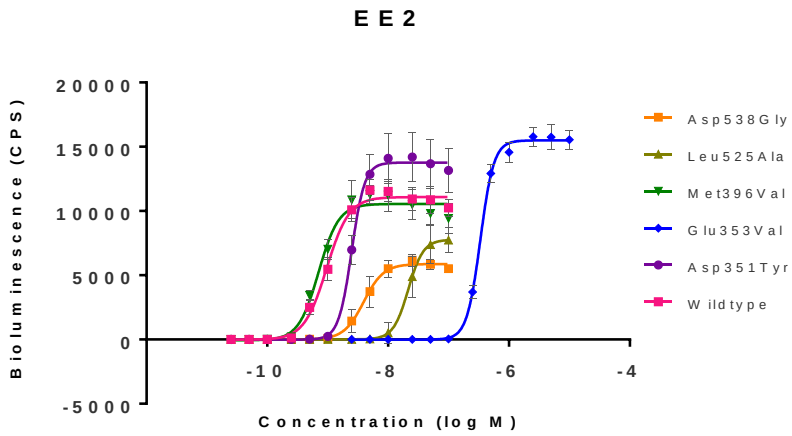
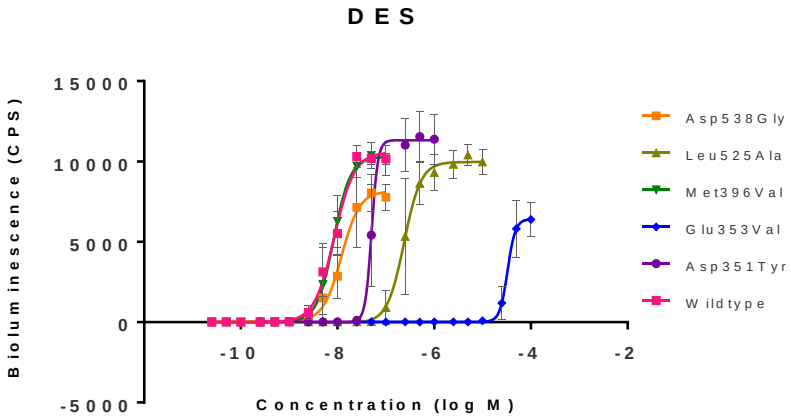

Asp538Gly
EC50 (log M)
10 0
10 -1
10 -2
10 -3
10 -4
10 -5
10 -6
10 -7
10 -8
10 -9
10 -10
Wildtype
Asp538Gly
E2
Nonylphenol
S-equol
Genistein
Coumestrol
DES
EE2
BPA
Mestranol
Tamoxifen
Daidzein
Resveratrol

Fig 4.10. Dose-response curves of 12 tested ligands evaluated by wildtype and mutant hERα yeast estrogen bioreporters. (Values are presented as mean±SD, n = 6, dose-response curves are fitted 4 parameters logistic curves.)


E2
Bioluminescence (CPS)
20000
15000
10000
5000
0
-5000
-10
-8
-6
-4
-2
Concentration (log M)
Asp538Gly
Leu525Ala
Met396Val
Glu353Val
Asp351Tyr
Wildtype


EE2
Bioluminescence (CPS)
20000
15000
10000
5000
0
-5000
-10
-8
-6
-4
Concentration (log M)
Asp538Gly
Leu525Ala
Met396Val
Glu353Val
Asp351Tyr
Wildtype


DES
Bioluminescence (CPS)
15000
10000
5000
0
-5000
-10
-8
-6
-4
-2
Concentration (log M)
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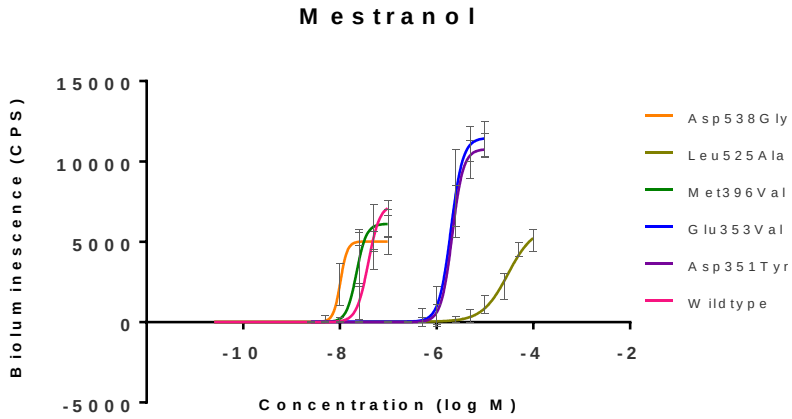
Leu525Ala
Met396Val
Glu353Val
Asp351Tyr
Wildtype


Mestranol
Bioluminescence (CPS)
15000
10000
5000
0
-5000
-10
-8
-6
-4
-2
Concentration (log M)
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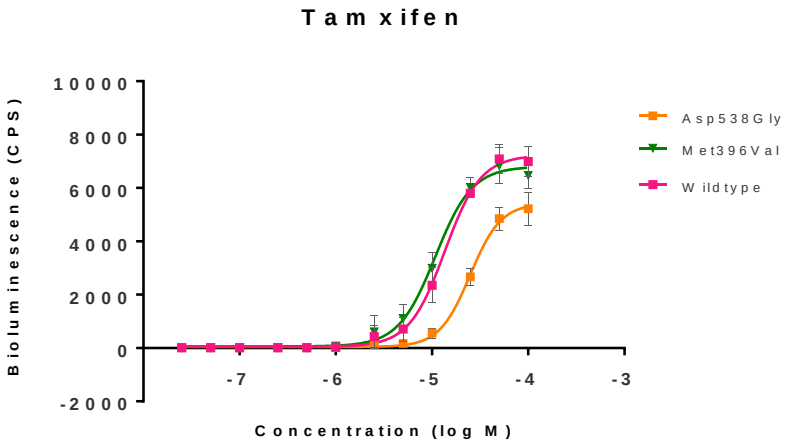
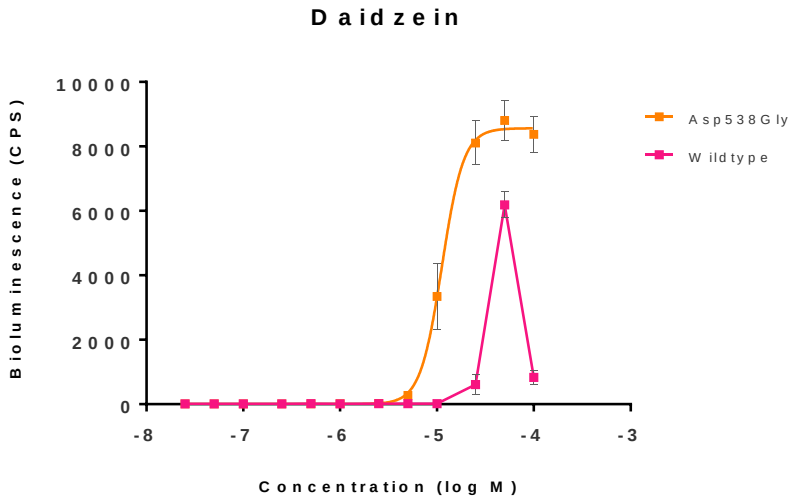
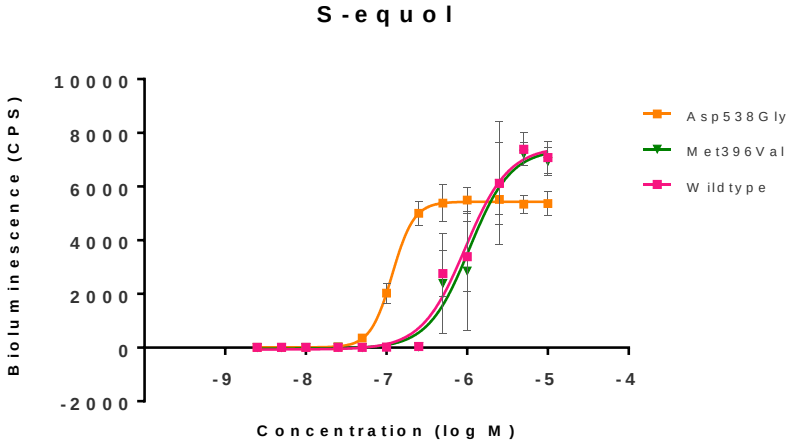
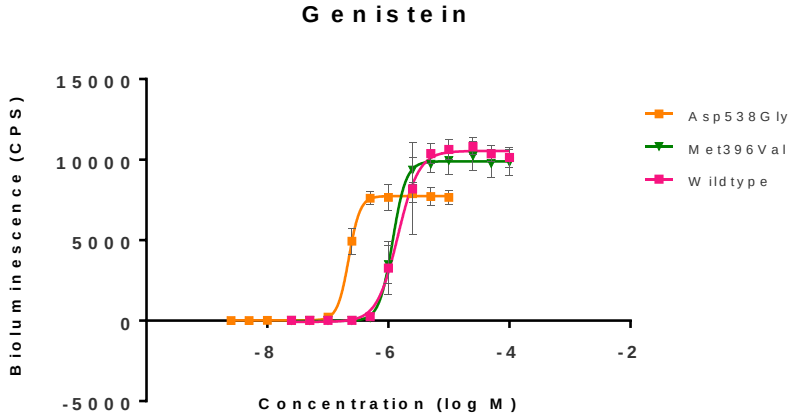
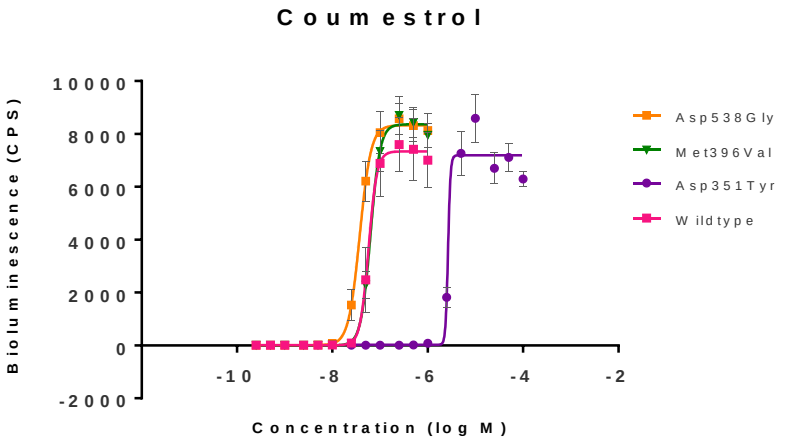
Leu525Ala
Met396Val
Glu353Val
Asp351Tyr
Wildtype

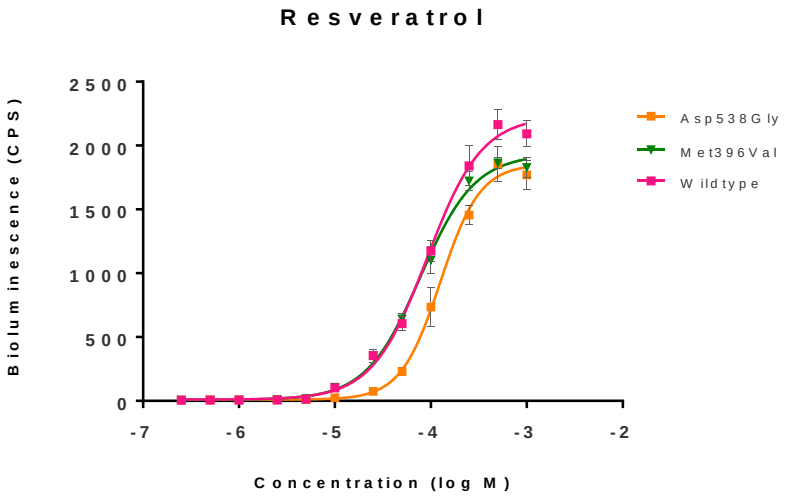

Tamxifen
Bioluminescence (CPS)
10000
8000
6000
4000
2000
0
-2000
-7
-6
-5
-4
-3
Concentration (log M)
Asp538Gly
Met396Val
Wildtype

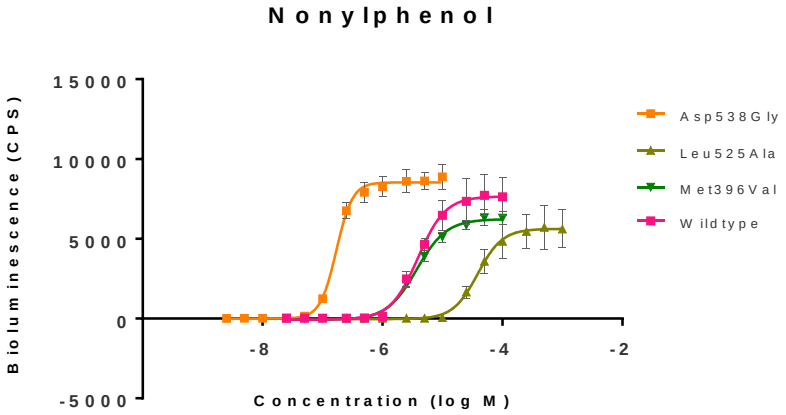

Daidzein
Bioluminescence (CPS)
10000
8000
6000
4000
2000
0
-8
-7
-6
-5
-4
-3
Concentration (log M)
Asp538Gly
Wildtype

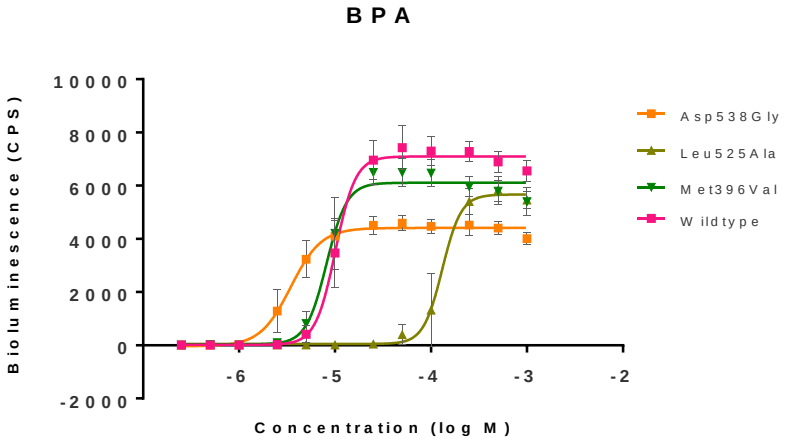

S-equol
Bioluminescence (CPS)
10000
8000
6000
4000
2000
0
-2000
-9
-8
-7
-6
-5
-4
Concentration (log M)
Asp538Gly
Met396Val
Wildtype


Genistein
Bioluminescence (CPS)
15000
10000
5000
0
-5000
-8
-6
-4
-2
Concentration (log M)
Asp538Gly
Met396Val
Wildtype


Coumestrol
Bioluminescence (CPS)
10000
8000
6000
4000
2000
0
-2000
-10
-8
-6
-4
-2
Concentration (log M)
Asp538Gly
Met396Val
Asp351Tyr
Wildtype


Resveratrol
Bioluminescence (CPS)
2500
2000
1500
1000
500
0
-7
-6
-5
-4
-3
-2
Concentration (log M)
Asp538Gly
Met396Val
Wildtype


Nonylphenol
Bioluminescence (CPS)
15000
10000
5000
0
-5000
-8
-6
-4
-2
Concentration (log M)
Asp538Gly
Leu525Ala
Met396Val
Wildtype


BPA
Bioluminescence (CPS)
10000
8000
6000
4000
2000
0
-2000
-6
-5
-4
-3
-2
Concentration (log M)
Asp538Gly
Leu525Ala
Met396Val
Wildtype

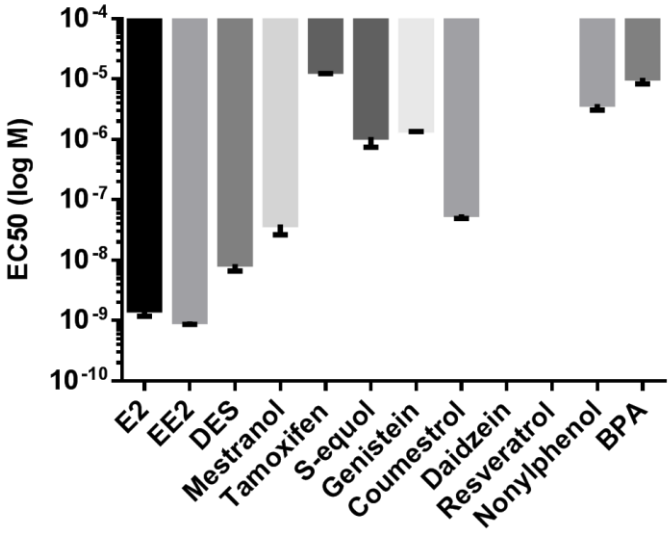


Fig 4.11. Wildtype ERwt-pCYC strain's EC50 response to 12 estrogenic compounds

Response of mutant strains ER351-pCYC, ER353-pCYC, ER396-pCYC, ER525-pCYC, ER538-pCYC to 17β-estradiol

The effect the five hERα mutations were firstly evaluated by exposing the mutant carrying yeast bioreporters to serial diluted natural estrogen 17β-estradiol. The dose response curves of the wildtype and mutant yeast bioreporters are shown in Fig 4.12. EC50 values for each bioreporter are presented in Fig 4.13 and Table 4.7.

Among the five mutants, three of them demonstrated statistically different responses to 17β-estradiol compared to the wildtype. Asp351Tyr mutant demonstrated moderately reduced response, reporting EC50 of (5.09±0.60)E-09 M, compared to the wildtype's EC50 of (1.51±0.32)E-09 M. Glu353Val demonstrated the weakest response, reporting EC50 of (1.90±0.26)E-06 M, three magnitude lower than the wildtype. Leu525Ala also demonstrated considerable decrease in the response, with EC50 of (3.82±1.03)E-08, about one magnitude lower than the wildtype. Two of the mutants, Met396Val and Asp538Gly reported EC50 of (1.69±0.81)E-09 M and (1.14±0.04)E-09 M respectively, which had with no statistical significance compared to the wildtype.

Response of Asp351Tyr mutant to estrogenic EDCs

As Asp351Tyr mutant bioreporter resulted in reduced response to E2 compared to the wildtype, this mutation also consistently resulted in significantly decreased responses when exposed to the other 11 tested estrogenic EDCs (Table 4.7). For less potent compounds including nonylphenol, S-equol, tamoxifen, daidzein and resveratrol, no detectable response was present. When exposed to genistein and BPA, Asp351Tyr mutant produced detectable yet not strong enough

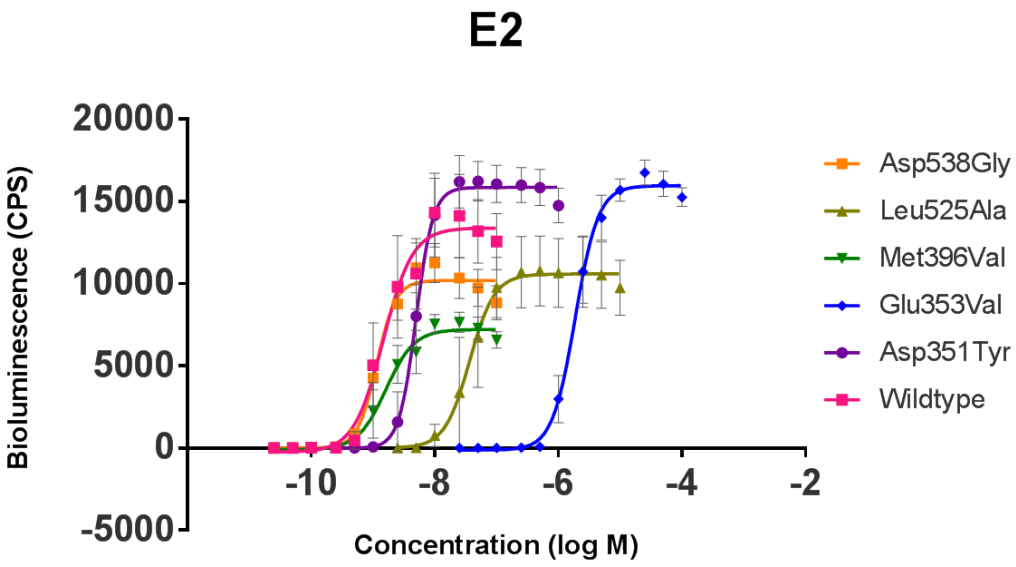


Fig 4.12. Dose-response curve of hERα wildtype and mutant yeast bioreporters to serial diluted 17β-estradiol. Values are Mean±SD, n=6.

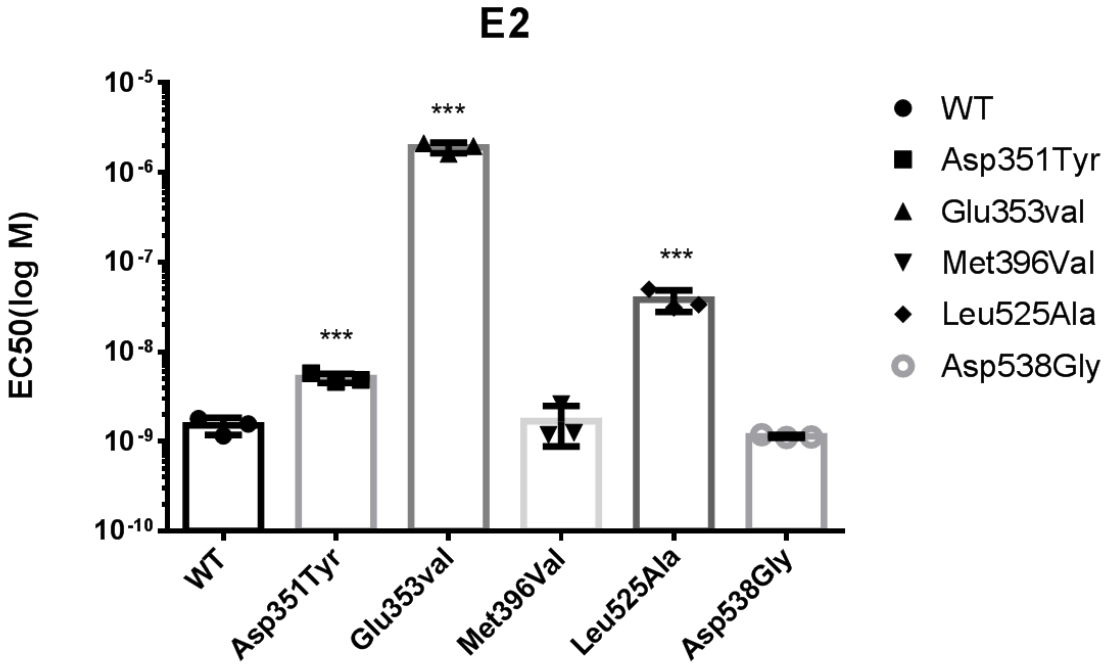


Fig 4.13. EC50s of hERα wildtype and mutant bioreporters in response to 17β-estradiol. (Mean±SD, ***: p<0.001)

response to properly report EC50. EE2 exposure produced the strongest response, with EC50 of (2.49±0.03)E-09 M, slightly lower than the wildtype EC50 of (9.68±1.06)E-10 M. DES, mestranol and coumestrol exhibited sharper decrease in the response, with the EC50 of DES shifted about 1 magnitude, mestranol and coumestrol shifted around 2 magnitude.

Asp351Tyr was initially identified as a natural point mutation in an antiestrogen resistant (tamoxifen-stimulated) human breast tumor cell line(Wolf and Jordan 1994). Several studies have found that Asp351Tyr mutation can change the behavior of antiestrogens including tamoxifen and raloxifene from antagonistic to agonistic and trigger estrogenic responses (Catherino and Jordan 1995, Catherino, Wolf et al. 1995, Levenson, Catherino et al. 1997, Levenson and Jordan 1998). Asp351Tyr is located close but not inside the binding pocket. Previous studies have suggested that Asp351 residue could be involved in stabilizing Helix-12 of hER ligand binding domain to seal the binding pocket so that the estrogen receptor can be activated. Mutating Asp351 to Tyr changes the side chain from negative charged to hydrophobic, which could reduce its interaction with Helix12, making helix 12 less prone to the closed position, hence causing reduced transcriptional activity. This may explain why in this yeast bioreporter, all tested chemicals exhibited reduced EC50.

Response of Glu353Val mutant to estrogenic EDCs

Glu353 is the residue in hER ligand binding pocket responsible for ligand recognition and providing electrostatic interaction with ligands including E2, EE2 etc. Previous studies demonstrated that mutation of Glu353 will significantly reduce the binding affinity of E2, leading to reduced receptor activation(Brzozowski, Pike et al. 1997, Ekena, Katzenellenbogen et al. 1998).

In our study, 8 of the 12 tested ligands failed to produce any detectable response with Glu353Val mutant. Only 4 ligands including E2, EE2, DES and Mestranol triggered significantly weaker response compared to the wild type. Compared to the wildtype, the EC50 of EE2 sharply increased from (9.68±1.06)E-10 M to (3.36±0.27)E-07M; DES and mestranol demonstrated similarly inhibited responses, each had EC50 increased from (8.67±1.99)E-09 M to (3.36±0.45)E-05 M and (3.93±1.28)E-08 M to (1.99±0.16)E-06 M, respectively.

Mutating Glu to Val changes the negatively charged side chain to hydrophobic side chain, eliminating the potential electrostatic interaction between this residue and the ligand. Therefore any ligand that relies on this interaction to properly dock into the binding pocket could suffer from weaker binding. In our study, all tested ligands showed no or drastically reduced response, suggesting that Glu353 residue plays pivotal role for effective binding by not only the natural estrogen E2, but also other tested EDCs.

Response of Met396Val mutant to estrogenic EDCs

Met396Val mutation was initially identified on a primary breast cancer tumor, and no effect were found by the time it was reported(Roodi, Bailey et al. 1995). Met396 residue does not reside in the binding pocket, but is close to another critical residue Arg394. Since Met and Val both have hydrophobic side chain, this mutation does not cause a significant change to the local hydrophobic environment.

In this study, Met396Val mutant showed detectable responses to all 12 ligands at similar level as the wild type (Table 4.7). No significant difference could be determined between the EC50 of

Met396Val and that of wildtype for any of the 12 ligands, suggesting that Met396Val mutation does not change the ligand binding and transcriptional activation of hERα.

Response of Leu525Ala mutant to estrogenic EDCs

Leu525 is a residue in the ligand binding pocket of hERα that can have direct contact with the ligand. Previous study found that mutating the Leu525 to Ala can lead to inhibited estrogen binding and transcriptional activity(Kirk, Karen et al. 1996). In this study, Leu525Ala mutant strain did not respond to the weaker ligands including daidzein, S-equol, coumestrol, resveratrol. Genistein, though less estrogenic than coumestrol when tested by the wildtype bioreporter, produced weak detectable response by Leu525Ala mutant (Table 4.7). Tamoxifen also produced weak detectable response, but EC50 was not reportable. E2, EE2, DES, nonylphenol and BPA exposure all produced significantly decreased responses, with each EC50 increased ~ 1 magnitude than that of the wildtype. Leu525Ala mutation caused the most changes in response to mestranol, which had EC50 increased ~3 magnitude, from (3.93±1.28)E-08 M to (3.07±0.36)E-05 M. Overall, Leu525Ala mutation lead to inhibited response for all ligands compared to the wildtype, suggesting that Leu525 plays an critical role in hERα's ligand binding and transcriptional activation function. Unlike other critical residues such as Glu353, Arg394, or His594, Leu525 has a hydrophobic side chain and doesn't provide electrostatic charge to the ligand. However Leu535 is positioned on the exit of the binding pocket, help shaping the binding cavity with its hydrophobic sidechain. When Leu525Ala mutation occurs, this cavity is likely to be more open, making ligands harder to stay/bind in the binding pocket. Depends on the shape of the ligand, Leu525 may or may not be involved in the direct contact with the ligand. This may explain why

Leu525Ala caused decreased transcriptional activity in the yeast reporter with different level of reduction for different ligands.

Response of Asp538Gly mutant to estrogenic EDCs

In this study, Asp538Gly mutation caused both increased and decreased response to different ligands (Table 4.7). When exposed to natural estrogen E2, Asp538Gly mutant showed similar response compared the wild type, with the EC50 of (1.14±0.04)E-09 M versus (1.51±0.32)E-09 M for the wild type. EE2, DES and tamoxifen triggered lower response in Asp538Gly mutant than the wildtype, with EC50 shifted from (9.68±1.06)E-10 M to (4.01±0.74)E-09 M, (8.67±1.99)E-09 M to (1.24±0.16)E-08 M and (1.36±0.12)E-05 M to (2.50±0.17)E-05 M respectively. Interestingly, for the rest of tested EDCs including mestranol, BPA, daidzein, S-equol, genistein, coumestrol, and nonylphenol, Asp538Gly mutant produced significantly stronger response compared to the wild type strain. Notable response increase was observed for nonylphenol and S-equol, whose EC50 decreased from (3.87±0.79)E-06 M to (1.70±0.03)E-07 M and (1.10±0.36)E-06 M to (1.18±0.08)E-07 M respectively, about 1 magnitude increase of activity. The response triggered by daidzein increased from non-reportable response to EC50 of (1.13±0.06)E-05 M. Genistein also produced significantly increased response with EC50 shifted from (1.45±0.10)E-06 M to (2.23±0.06)E-07 M. BPA also posed significantly higher activity with Asp538Gly mutant, with EC50 shifted from (1.05±0.21)E-05 M to (3.52±0.62)E-06 M. Coumestrol demonstrated slightly increased response with EC50 shifted from (5.78±0.84)E-08 M to (3.76±0.34)E-08M.

Asp538Gly mutation was initially identified in primary breast tumor tissue by a large-scale cancer somatic mutation screening study and reported on COSMIC (Forbes, Bhamra et al. 2008, Kan,

Jaiswal et al. 2010). Later studies discovered that Asp538 residue has relatively higher mutation frequency in metastasis tumors (Alluri, Speers et al. 2014, Min, Aditya et al. 2014). The specific mutation Asp538Gly was found associated with acquired endocrine therapy resistance (tamoxifen resistance) in breast cancer patients who received long-term antiestrogen therapy(Robinson, Wu et al. 2013). Through MD simulation and *in vitro* ERE-luciferase mammalian bioreporters, previous studies suggested that this mutation force hERα binding domain to maintain in agonist-binding conformation, rendering the estrogen receptor constitutively active and hence causing anti-estrogen drugs ineffective. In our study, Asp538Gly mutant yeast bioreporter did not demonstrate constitutive transcriptional activity, as no constitutive bioluminescence were produced. Instead, Asp538Gly yeast bioreporter revealed an unusual response profile to different tested EDCs. Out of the 12 tested ligands, only 4 of them, E2, EE2, DES and resveratrol, elicited identical or reduced response compared to the wildtype, whereas the rest of 8 ligands all exhibited elevated potency when been exposed to Asp538Gly mutant. According to the finding of previous studies, Asp538Gly mutation could form hydrogen bond with Asp351 residue, which could help and induce the helix-12 of hER ligand binding domain to stay in the closed position. This stabilizing effect is likely to be responsible for the increased potency of tested chemicals in the yeast bioassay. In this study, higher degree of response increase was seen when less potent ligand was tested, suggesting the stabilizing effect makes bigger impact for weak estrogen receptor ligands. The ligands showing significant elevated potency with Asp538Gly mutant include common phytoestrogens, industrial compounds that are easily accessible either passively or proactively in daily life. The fact that Asp538Gly mutation causes elevated potency for these ligands in the yeast bioreporter raises health alert especially

for breast cancer patients harboring this mutation in either the primary or metastatic tumors. The advantage of yeast estrogen bioreporters is that estrogen receptor is the only nuclear receptor present in the cell, eliminating possible interference from other nuclear receptors. However although the yeast cell contains essential coregulators for estrogen receptor to properly exert transcriptional activation, yeast doesn't share the same cellular components as human cells. Therefore further testing in mammalian cells and animal model is warranted to verify the observed effect. In addition, special care should be taken in such tests when preparing the medium/nutritious supplements, as weak estrogenic compounds present in the medium/food could possibly cause unexpected estrogen receptor activation.

Comparison of hERα mutants' response profile to estrogenic EDCs

To compare the response profile of each mutant strain to the 12 tested ligands, hierarchical clustering was performed using the mean EC50 values obtained from the yeast bioassay (Fig 4.14). In hierarchical clustering, the response pattern for the wildtype is closest to Met396Val, which is in agreement with the finding that Met396Val didn't cause altered response to all 12 compounds. When comparing the response patterns for the other mutants, different mutation on hER produced a different response profile to the 12 chemicals, suggesting that the degree of response changes resulted from a mutation depends on the actual structure of individual EDC. E2 and the two synthetic estrogen EE2 and DES clustered together, suggesting their similar response pattern to the mutants. Compared to other weaker estrogenic EDCs, the response pattern of E2 was more different, indicating that hER mutant's response to E2 do not necessarily indicate similar response to other estrogenic EDCs. This is particularly notable for Asp538Gly mutant, as E2 didn't yield a significantly different response to this mutant, yet other weaker estrogenic EDCs all yielded

significantly stronger responses. These data together suggest that individual EDC testing for each hER mutation variant is necessary, since response trend cannot be predicted from a single chemical exposure. Considering that this study only tested 12 EDCs, it is reasonable to assume that broader range of chemicals may also exhibit such discrepancy.

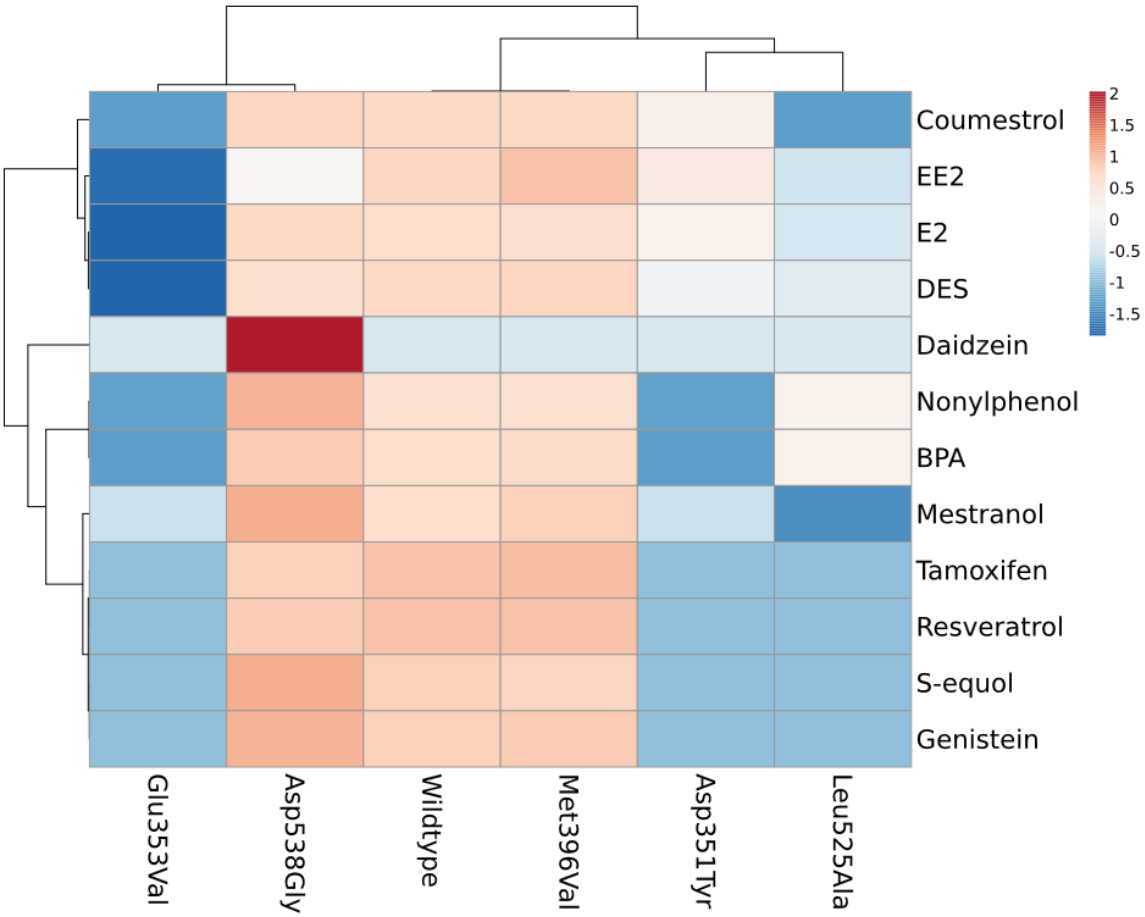


Fig 4.14. Two-way hierarchical clustering and heatmap of EC50 for all 12 ligands and 6 yeast bioreporters. Higher value represents higher potency (lower EC50 value). Two way hierarchical clustering was performed using ClustVis(Metsalu and Vilo 2015). Average EC50 values from the yeast assay were –log transformed and missing values were filled with 0.01 prior to the clustering. For hierarchical clustering, rows were centered; unit variance scaling was applied to rows. Both rows and columns were clustered using correlation distance and Ward linkage.

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Chapter 5. Conclusions and Future Perspectives

The prevalence of EDCs in the environment and their health risks urge a thorough understanding on the fate, transport and transformation of EDCs in the environment, and how they can affect human and animal health. The studies in this dissertation were intended to add further knowledge on these central topics of EDCs, and specifically focused on the application of yeast bioluminescent reporters to study biotransformation of EDCs in wastewater treatment, and the interaction between human estrogen receptor mutants and EDCs with both computational modelling and experimental approach.

Chapter 2 demonstrated the use of the standardized yeast bioluminescent assays as a high-throughput screening tools to monitor the estrogenic, androgenic potencies and toxicity in the wastewater treatment systems. To evaluate the sensitivity and reproducibility of the yeast assay in wastewater monitoring, 7 days semi-continuous batch reactor experiments was conducted using the activated sludge and raw wastewater spiked with hormones. Yeast bioreporter assay successfully captured the rapid removal of estrogenic and androgenic activities in the bioreactors, and also demonstrated good reproducibility and sensitivity. This standardized assay was then applied in a 12 month comparative study on the wastewater effluent from the parallel-operated MBR and TAS located at Powell, TN, USA. The yeast assay detected no significant androgenic response in all tested TAS and MBR samples. While all TAS effluent samples and most (3 of the 31 dates) MBR effluent samples showed detectable estrogenic activity. The EEQ_{50} for TAS effluent ranged from 21.61 ng/L to 0.04 pg/L and averaged 3.25 ng/L. For MBR, the effluent EEQ ranged from 2.88 ng/L to 0.0134 pg/L and averaged ~10 fold less (0.32 ng/L) than TAS. Despite the large temporal variation, the EEQ level in MBR effluent was consistently lower than that of TAS on any given sampling day. Most MBR effluent samples also contained less cytotoxicity than TAS,

suggesting that MBR can significantly reduce the release of EDCs and toxic compounds into the environment. Correlation analysis was also conducted and revealed no significant correlation between effluent EEQ level and WWTP operational parameters including MLSS, SRT, HRT and BOD. This chapter also discussed the common issues in environmental sample testing caused by sample toxicity, and suggested that EC_{20} could be a more appropriate metrics than EC_{50} in environmental sampling. Overall, this chapter demonstrates with current state-of-the-art wastewater treatment technology, trace level of EDCs are still been discharged into the aquatic environment continuously. This chapter also suggests that the yeast bioluminescent assay is suitable to serve as a sensitive, high-throughput, rapid and low-cost EDCs screening tool for routine wastewater monitoring purpose.

Chapter 3 and 4 investigated the interaction of environmental EDCs with five human estrogen receptor mutants at molecular level through computational modelling and experimental bioassay. These mutations are either inside or close to the ligand binding pocket of the protein, and were selected from cancer mutations database and previous literature. Chapter 3 specifically examined the interaction of the *in silico* wildtype and mutant model with 29 selected environmental EDCs. Ensemble docking of selected MD conformations of wildtype hERα ligand binding domain showed moderate correlation with previous experimental binding assay data, suggesting a relatively good performance of the docking scheme, especially considering the evaluated ligands were strong to medium ER binders. When comparing the docking results between hERα wildtype and the five mutants, ensemble docking simulation predicted decrease of binding affinity for nearly all 29 ligands when the tested five hERα mutations occur. Among the five hER mutations, Glu353Val was predicted to yield the highest reduction of binding affinity,

while Leu525Ala was predicted to yield the least reduction, with some ligands demonstrated no change or slightly increase in binding affinity. The study in chapter 3 was attempting to test whether computational modelling can provide informative guidance for experimental testing of estrogen receptor mutants' response to EDCs. In this study, the difference between 2B1Z wildtype and mutant docking scores was very modest, the accuracy of the predictions requires further validation through experimental mutagenesis study.

In chapter 4, a new yeast estrogen bioluminescent reporter construct was established and optimized for estrogen receptor mutation study. Through site-directed mutagenesis, five yeast bioreporters harboring separately the five selected hER mutations were constructed. A list of 12 EDCs including the natural estrogen 17β-estradiol (E2), synthetic hormones, plasticizer, phytoestrogen and industrial compound were evaluated with the wildtype and mutant hER yeast bioreporters. Out of the five mutants, Glu353Val mutant showed the most drastic decrease in the response to all 12 EDCs compared to the wildtype, further confirming the critical role of Glu353 for ligand-receptor interaction. Met396Val didn't show any significant difference with the wildtype in response to the 12 EDCs. Asp351Tyr and Leu525Ala showed moderately decreased response to tested EDCs compared to the wildtype. Compared to the other mutants, Asp538Gly produced an interesting response to these EDCs. Out of the 12 tested EDCs, only 4 of them, E2, EE2, DES and resveratrol, elicited identical or reduced response compared to the wildtype, whereas the rest of 8 ligands all exhibited elevated potency when been exposed to Asp538Gly mutant. The EDCs showing significant elevated potency with Asp538Gly mutant include common phytoestrogens, industrial compounds that are easily accessible either passively or proactively in daily life. The fact that Asp538Gly mutation causes elevated potency for these ligands in the yeast

bioreporter raises health alert especially for breast cancer patients due to the high frequency of this mutation in breast cancer metastatic tumors. When comparing the response patterns of mutant and wildtype bioreporter to the 12 EDCs, different mutations on hER produced different response profiles, suggesting that the degree of response changes resulted from a mutation depends on the actual structure of individual EDCs. In addition, the response trend of hER mutants for broader spectrum of EDCs may not be readily predictable from the response of the natural estrogen or other individual EDC. Therefore individual EDC screening for each hER mutation variant is necessary.

The advantage of yeast estrogen bioreporters is that estrogen receptor is the only nuclear receptor present in the cell, eliminating possible interference from other nuclear receptors. However although the yeast cell contains essential coregulators for estrogen receptor to properly exert transcriptional activation, yeast doesn't share the same cellular components as human cells. Therefore further testing in mammalian cells and animal model is warranted to verify the observed effect of hER mutations. In addition, special care should be taken in such tests when preparing the medium/nutritious supplements, as weak estrogenic compounds present in the medium/food could possibly cause unexpected estrogen receptor activation.

When comparing the results form computational modelling and the yeast bioassay, the agreement between these two approaches seem to depend on the location of the mutation on hER. For residues inside the binding pocket or have direct contact with the ligand (i.e. Glu353 and Leu525), molecular docking predicted similar trends as what was demonstrated in the yeast bioassay. Yet for residues outside the binding pocket such as Asp351 and Asp538, computational simulation failed to report results with good agreement with yeast bioassay. Part of the reason

is that changes of transcriptional activity, as tested in the yeast bioassay, is determined not only by ligand binding affinity, but also interaction with other regulatory proteins. To further decipher the mode of action for estrogen receptor, further understanding on two main aspects are needed. Firstly, the structural basis of the whole ER protein, including both the N-terminal domains and C-terminal domains, and the interaction between the domains should be addressed. Secondly, the type and structure of coregulators, machinery of interactions between ER and coregulators in different cell type needs to be elicited as well. As the function estrogen receptor involves a series of complex interactions, these knowledge could also bring new therapeutic targets for estrogen receptor related disease.

Vita

Jun Wang was born on Sep 23, 1986 at a small village Xiangyang, the rural area of Nanjing, China. He grew up in the small village and went to the downtown area of Nanjing at the age of 11 for the last year of elementary school at Shigu Road Elementary School. Jun then attended the 24th middle school in 1998 and the High School affiliated to Nanjing Normal University in 2001. He finished his bachelor degree in biological sciences at Nanjing Agricultural University in 2008, and came to University of Tennessee for doctoral study in microbiology in the same year. Jun has strong interests in both science and art since very little. These childhood interests continue to grow in his life. He learned accordion for 2 years at the age of 10. He is also a self-taught guitarist and played in a band during college. Jun also has strong interest in visual arts, especially photography and videography. In May 2015, Jun married Xiaoxin Liu at Lenoir City, TN, witnessed by their Pastor Dr. Changwen Chen.