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Engineered Nuclear Hormone Receptor-Biosensors for Environmental Monitoring and Early Drug Discovery

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1. Introduction

Bacterial Biosensors are engineered microorganisms that can be used to detect a variety of chemicals. These chemicals can include heavy metals, toxins, hormones, hormone-like drugs and environmental endocrine-disrupting pollutants. In general, bacterial biosensors are engineered to express a biosensing protein, which can selectively bind to a target chemical (usually referred to as a “ligand”). When the target ligand is present, the biosensor protein produces an easily readable change in the cell behaviour. For example, the biosensing protein may produce a change in fluorescence or enzyme activity, or as shown in Fig. 1 & 2, may change the growth rate of the expressing cell when an appropriate ligand is present (Gillies et al, 2008; Skretas et al, 2007; Skretas & Wood, 2005a, 2005b, 2005c).

Fig. 1. Growth dependent bacterial biosensor cell. A reporter protein gene is contained on a carrier plasmid, which is transformed into a microbial strain. The expressed biosensor protein produces a ligand-sensitive growth phenotype. In this case, the presence of the appropriate ligand for the biosensor protein increases the growth rate of the biosensor cells.

The bacterial biosensors described in this chapter have been developed specifically for detecting and identifying chemicals that target human and animal nuclear hormone receptors (NHRs). As such, they can be used for identifying potentially valuable drugs for
treating a variety of cancers and metabolic disorders, or they can be used to detect and identify pathogenic environmental chemicals that act through various NHRs. In drug discovery, the link between chemicals binding to NHRs and various disease states is recognized across many different metazoans (Hu et al, 2008; Jofre & Karasov, 2008).

Fig. 2. (a) Schematic representation of the NHR biosensor protein and related growth phenotypes (Gillies et al, 2008; Skretas et al, 2007; Skretas & Wood, 2005a). The activity of the TS reporter enzyme is dependent on the configuration of the allosteric sensor protein, which is modulated by the binding of an NHR ligand. The activity of TS affects bacterial DNA synthesis and cellular metabolism (b). The resulting change in growth phenotype can be quantified by optical absorbance at 600 nm in liquid growth medium, allowing an indirect determination of the ligand’s agonistic or antagonistic behaviour. Abbreviations: ΔI-SM (mini-intein splicing domain); MDB-maltose binding domain; TS-thymidylate synthase; dUMP- deoxyuridine monophosphate; dTMP- deoxythymidine monophosphate; FdUMP-5-fluoro-2’-deoxyuridine 5’-monophosphate; NADP- nicotinamide adenine dinucleotide phosphate; LBD-ligand binding domain of nuclear hormone receptor.
In humans, aberrant NHR binding of native and other hormone-like compounds is associated with a wide variety of disorders (Grycewicz & Cypryk, 2008), including dyslipidemia, hypogonadism, endometriosis, cancer, obesity and diabetes, as well as reproductive organ dysfunction and infertility (Feldman et al., 2008; Fessler, 2008; Malm et al., 2007; Mattsson & Olsson, 2007; Ohno, 2008; Tancevski et al., 2009; Tokumoto et al., 2007). Mitigation of these and other disorders, however, can also be accomplished through NHR manipulation, where hormone-like compounds can reverse or otherwise treat a wide variety of diseases. Similar pathogenic NHR binding effects can be seen in animals, where hormonal imbalances arise from environmental endocrine disrupting compounds (EDCs), such as pollutants and insecticides. These imbalances can lead to infertile egg production, tissue abnormalities, degraded gonadal structure, demasculization, altered species metamorphosis patterns and abnormally fast growth (Fernandez et al., 2007; Hu et al., 2008; Katsu et al., 2007; Rempel & Schlenk, 2008). For this reason, identification of EDCs and environmental screening for endocrine disrupting activity is a critical application as well.

In humans, there are six major NHR groups, the best studied of which include the Estrogen Receptor (ER-like), Thyroid Hormone Receptor (TR-like) and Retinoid X Receptor (RXR-like) (Doweyko, 2007). Inside the cells, these NHRs bind to DNA and various transcriptional co-activators and co-repressors to regulate the transcription of large numbers of genes in response to their hormone ligands. This ability gives NHRs a tremendous impact on cell maturation, metabolism and homeostasis (Acosta-Martinez et al., 2007; Baxter & Webb, 2009; Brettes & Mathelin, 2008).

A key element of the NHRs is that, in addition to their native hormones, they can bind to a wide variety of endocrine disruptors (EDs) and complex pharmaceuticals (Fig. 3). Further, several subtypes can exist for a given NHR family (e.g., ERα or ERβ, and TRα or TRβ). Environmental pollutant EDs that target NHRs include BPA, PCBs, and dioxins, while endocrine active compounds in foods can include vitamins, phospholipids, phytoestrogens and fatty acids. Many pharmaceuticals have been developed to target NHRs, with the most important compounds typically exhibiting highly subtype-selective binding within an NHR group. Notable examples include the Selective Estrogen Receptor Modulators (SERMs; e.g. Raloxifene and Tamoxifen), and the Selective Thyroid Hormone Receptor Modulator (STRM; e.g. Eprotirome, currently in Phase II clinical trials) (Baxter et al., 2004; Leung et al., 2007). The NHR proteins can also form homo- or hetero-dimers and tetramers within the NHR subclasses (e.g., ER-ER; ER-RXR), and can form various combinations of subtype homo- and heterodimers (e.g., ERα-ERβ dimer). These aspects of NHR action can greatly complicate their function in various cells and organs, leading to a wide variety of tissue-specific effects in response to ligands of various classes. The similar structures and functions of the NHRs makes them a perfect fit for engineering biosensors, especially since they can be expressed well in bacteria or yeast cells. Additionally, the mechanism by which ligand binding triggers gene expression is well known, which has made NHRs and NHR LBDs highly tractable for drug discovery and environmental screening in high throughput systems. There are two basic classifications for compounds that bind to NHRs: agonists and antagonists. In general, agonist compounds tend to trigger hormone-related gene transcription, while antagonists tend to suppress transcription. The exact response of a cell to a given endocrine active compound, however, depends on a variety of factors, which include the presence of various co-activators and co-repressors and aspects of the metabolic state of the cell. At the molecular level, the primary determinant for the differential response of the NHR to these two types of compounds is the...
repositioning of a conserved helix, generally known as helix-12 (Fig. 4a), upon ligand binding (Gulla & Budil, 2007; Shiau et al, 2002). When the bound ligand is an agonist, helix-12 tends to shift towards the NHR binding pocket, creating a charged area on the protein surface. This surface is then occupied by a co-activator, which results in initiation of transcription (MacGregor & Jordan, 1998; Schapira et al, 2000; Shiau et al, 1998). Antagonists are commonly equipped with bulky functional side group(s), causing helix-12 to rotate

Fig. 3. Selected structures of compounds binding to NHRs. Thyroid receptor ligands include the compounds T₃ (a natural TR agonist), TRIAC (a natural TR agonist), KB-141 (a synthetic TRβ-selective agonist) and GC-1 (a synthetic TRβ-selective agonist), while estrogen receptors bind tamoxifen (a subtype-selective ER modulator), raloxifene (a subtype-selective ER modulator), benzophenone-2 (an ER agonist found in many cosmetics and perfumes), GW7604 (a synthetic selective ER downregulator), EM652 (a synthetic selective ER downregulator), E₂ (17-β-estradiol – the native ER ligand), BPA (an ER agonist and suspected ER-disruptor found in many consumer plastics), and DES (an ER agonist, formerly available small-molecule therapeutic which has been linked to cervical cancer).
away from the binding pocket, which typically results in suppression of transcription (Fig. 4a; Koehler et al, 2005).

Fig. 4. (a) Comparison of ER ligand binding domain structures, with a focus on helix-12 repositioning in response to agonist binding (left; ERα bound to genistein, PDB ID: 1X7R) or antagonist (right; ERβ bound to 4-hydroxytamoxifen, or Nolvadex®, a common drug used in treatment for breast cancer patients, PDB ID: 3ERT). The solvent accessible surface around 4-hydroxytamoxifen (structure shown in Fig. 3) is underlined in yellow on the right side of the compound. Genistein is inside of the active pocket, hidden behind the α-helices. Upon antagonist binding to ER, helix-12 rotates away from the binding pocket due to the antagonist’s extended functional group. This results in a change of the protein surface, making it inaccessible to co-activators. (b) Schematic representation of the NHR domains A through F (Hewitt & Korach, 2002; Norris et al, 1997). Abbreviations: AF-1 = Activation Function-1; AF-2a = Activation Function-2a; AF2 = Activation Function-2; DBD = DNA Binding Domain.

2. Engineered allosteric bacterial biosensor

In any screening process, the success of finding unique and active compounds depends greatly on the sensitivity of the method. A large diversity of available target proteins for screening is also essential, especially when searching for subtype-selective agonistic and antagonistic behaviours. Additionally, assay limitations must also be considered, such as the impacts of solvents used for delivering the test compounds, as well as growth media or temperature. These aspects of the assay can greatly affect the numbers of false positive and false negative results, as well as the reproducibility and robustness of the assay. Finally, for high throughput applications in large library drug screening, the assay must be simple, economical, and amenable to full or partial automation.
To generate bacterial biosensors for detecting hormones and hormone-like compounds, we have engineered the ligand-binding domains of various NHR proteins into an allosteric biosensor protein scaffold. The biosensor scaffold is composed of four protein domains, including a maltose binding domain, an intein stabilization domain, an NHR ligand-binding domain (LBD), and a thymidylate synthase (TS) reporter enzyme (Fig. 2). This scaffold is designed to link ligand binding by the NHR LBD to the activity of the fused TS reporter protein. Thymidylate synthase is a critical enzyme involved in bacterial DNA synthesis, which produces a strong growth phenotype on thymineless medium based on its activity. Specifically, the TS protein is part of bacterial folate metabolism, where it consumes a single methylene tetrahydrofolate molecule as it converts a single molecule of dUMP to dTMP. The dTMP is then used for DNA synthesis, which is required for cell growth. In practice, the chimeric sensor protein undergoes a structural change when an appropriate ligand is bound to the LBD, which alters the activity of the fused TS domain, and leads to a change in growth rate of the expressing bacterial cell (Fig. 2). The involvement of TS in the folate cycle allows both positive and negative selections for TS activity. Further, the stringency of the selection can be tuned by modulating the incubation temperature and concentrations of the antibiotic trimethoprim (Belfort & Pedersen-Lane, 1984; Gillies et al, 2008; Skretas et al, 2007; Skretas & Wood, 2005a). Although there are some differences between species, NHRs share similar domain structures and sequences within the subgroups, which have allowed new NHR biosensors to be generated using the same basic sensor protein scaffold. The biosensing microbial strains expressing these proteins are referred to as “Bacterial Biosensors”.

The NHR bacterial biosensors can be used to screen uncharacterized compounds for their effects on a variety of NHR targets. In particular, this method can be used for the detection and differentiation of agonistic and antagonistic compounds, and can be used to determine the half-maximal effective concentrations (EC$_{50}$ and IC$_{50}$ values) for a given compound (Skretas et al, 2007; Skretas & Wood, 2005a). Additionally, these bacterial biosensors can detect NHR subtype selectivity of a given compound, as well as species selectivity when used with animal-based bacterial biosensors (Gierach et al, 2011). In this case, the simple incorporation of an animal NHR ligand-binding domain generates a sensor for detecting ligands against that species.

The recognition of agonistic or antagonistic behaviour is directly correlated with TS activity of the sensor, expressed as an increase or decrease of cell growth on selective growth medium (Skretas & Wood, 2005a). In this selection system, TS activity closely depends on the conformation of the LBD-ligand complex. Reduced activity of the TS reporter enzyme, which is observed in the absence of ligands, or with non-binding or antagonistic ligands, results in lower bacterial growth. High TS activity is elicited by agonist binding and can be detected by observing the rate of increase in culture optical density at 600 nm (OD$_{600}$) over time. Importantly, the temperature plays an important role in the sensitivity of biosensors, and is related to the stability of TS and the cellular demand for dTMP. Therefore, bacterial cell growth is typically carried out in 96-well plates, incubated at precisely 34°C in a controlled humidity air shaker at 150 rpm (Gawrys et al, 2009; Gierach et al, 2011).

**Key features of the bacterial biosensors**

1. Each biosensor protein contains: Maltose Binding Protein-Intein-T4 Thymidylate Synthase Enzyme, with the NHR LBD inserted into the intein domain.
2. The activity of the fused TS enzyme is modulated by the amount and potency of the NHR ligand present.
3. TS activity can be detected qualitatively by colony formation on selective agar medium, or both qualitatively and quantitatively by observing changes in liquid growth medium optical density at 600 nm wavelength over time.

4. The LBD domain of one biosensor protein can be easily replaced with an alternate LBD, which has allowed the construction of functional biosensors for human ERα, ERβ, TRα, TRβ, PPARγ, fish (sole) ERβ and porcine (domestic pig) ERβ.

5. The assay method is non-radioactive, and has been developed for high throughput screening (HTS).

6. This method can detect strong ligands at low nM concentrations.

7. Weakly bound ligands, such as BPA and Tamoxifen for the human estrogen receptor, can be detected at low µM concentrations.

8. Three steps are required to complete a single set of assays: (a) overnight growth of fresh cells in non-selective LB medium; (b) dilution of the cells into a selective thymineless medium (–THY medium) and addition of the diluted test compounds and controls (this is done robotically in the HTS format); and (c) growth of cultures for 10 to 20 hours at 34°C, 150 rpm agitation and controlled humidity.

9. The limitations of the method are: the biosensor is sensitive to the presence of detergents, high levels of alcohols, solvents, and lipids (or any generally cytotoxic condition). Therefore, the final solvent concentration used in the assay should not exceed 1% DMSO or ethanol (test compound vehicle).

2.1 Construction of the bacterial biosensor strain

Vector and chimeric proteins

The biosensor protein is expressed from the pMal-c2 plasmid (New England Biolabs, Beverly, MA), where the plasmid backbone encodes the maltose binding protein (MBD) under control of the Ptac promoter. The biosensor gene is constructed through the following steps (Fig. 5): (1) the ∆I-SM gene, derived from the full-length *Mycobacterium tuberculosis* RecA intein (Wood et al, 1999), is fused to the C-terminus of the MBD; (2) the bacteriophage T4 td gene, encoding T4 TS reporter enzyme, is fused to the C-terminus of the ∆I-SM gene; (3) the native intein splicing activity is suppressed by mutation of the N- and C-terminal amino acids of the intein to alanine; (4) the NHR LBD is inserted into the ∆I mini-intein gene at the location where the original intein endonuclease domain was deleted; and (5) the Ptac promoter sequence is mutated to slightly increase basal expression of the overall fusion protein (Skretas & Wood, 2005b). The resulting plasmid names have the general form pMIT::[NHR], where pMIT stands for plasmid MBD-∆I intein-TS reporter scaffold, and [NHR] is the inserted LBD (Fig. 5). The constructed sensor plasmids are then transformed into the *E. coli* TS knockout strain D1210ΔthyA::KanR [FΔ(gpt-proA)62 leuB6 supE44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20 (Strr) xyl-5 mtl-1 recA13 lacIq] (Skretas et al, 2007).

A key component of the biosensor protein is the split ∆I-SM mini-intein domain, which is thought to increase the stability of the overall fusion and transduce binding information to the TS enzyme. Mutations at the N-terminus of the mini-intein (Cys→Ala) suppresses intein splicing, while the MBD was added to assure the solubility and increase the activity of the chimeric protein. Insertion of the NHR LBD is commonly accomplished by utilizing unique *AgeI* and *XhoI* restriction sites within the intein, which flank the intein-NHR insertion site (Skretas & Wood, 2005b). The constructed biosensors include two different mini-inteins for
LBD insertion: 110Δ383 and 96Δ400 (Wood et al, 1999). The 110Δ383 mini-intein was used more often, and includes the pMIT::ERβ*(h), pMIT::ERβ*(s), pMIT::ERβ*(p), pMIT::TRβ*(h) and pMIT::TRα*(h) biosensors, whereas the 96Δ400 intein was used for the pMIT::ERα*(h) fusion (Gierach et al, 2011). The specific mechanism of the biosensor action in bacterial cells is currently under investigation.

Fig. 5. Schematic representation of the plasmid vector pMal-c2 (top), which contains the MBD, intein, NHR LBD and TS genes. The swappable NHR LBD gene is inserted between the N- and C-terminal fragments of the intein. Several constructed biosensor protein fusion genes are also shown (bottom). In these diagrams, P\textsubscript{tac} = Ptac promoter for controlling biosensor protein expression; MBD = Maltose Binding Domain; N-Mtu = N-terminal segment (typically amino acids 1-110) of the ΔI-SM mini-intein; ER\textalpha, ER\textbeta, TR\textalpha, TR\textbeta = Estrogen Receptors alpha and beta, Thyroid Receptors alpha and beta, respectively, where (h) = human, (p) = porcine and (s) = sole; C-Mtu = C-terminal segment of the ΔI-SM mini-intein; TS = Thymidylate Synthase reporter enzyme. Plasmid names are shown on the right for each of the biosensor genes, where pMIT stands for plasmid MBD-ΔI-TS and the :: symbol indicates insertion of the indicated LBD.

2.2 Usage of the NHR bacterial biosensors
2.2.1 The High Throughput Screening (HTS)

The application of bacterial biosensors in a 96-well plate HTS format (Fig. 6) is based on an earlier protocol that employed glass culture tubes. The original glass culture tube method was very labor-intensive, and the number of samples and concentrations was limited by incubator space. Most importantly, large quantities of growth medium and ligands were

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needed for each experiment. The HTS method in 96-well plates is approximately 100 times more sensitive, and the cells, growth medium and ligands are dispensed by a robotic liquid handler (Biomek 2000, Beckman-Coulter), which assures greater mixing quality and repeatability.

2.2.2 Agonism and antagonism detection by biosensors

There are three major tests resulting in detection of ligand agonism and antagonism, as well as toxicity of the test compounds for bacterial cells (see Table 1). Compounds that stimulate the growth of cells in thymineless medium (-THY) are generally considered to be agonists, while antagonists can have no effect, or in some cases can decrease the growth of cells in –THY medium. Using estrogen receptor as an example, antagonist tests use competitive biosensor binding with estradiol (-THY+E$_2$ assay), where antagonist lowers TS activity relative to E$_2$ alone. However, in the TTM and TTM+E$_2$ assays the phenotypes are reversed, and low TS activity upon antagonist binding rescues cells. This reversal is very helpful in confirming LBD-specific effects, as opposed to more general metabolic effects. Toxicity can be determined by adding an uncharacterized compound to cells growing in non-selective medium (+THY), where cells grow freely in the presence of agonists or antagonists, but toxicity results in a loss of growth.

Fig. 6. Schematic representation of the intein-based biosensor method.

2.2.3 Sub-type receptor selectivity

The ligand binding domains of receptor subtypes are highly conserved (see Fig. 4), which allows multiple receptor subtypes to be used for biosensor generation. Differential binding to one biosensor subtype or another allows a quick determination of subtype selectivity for a given compound. In previous studies, we were able to confirm the subtype-selectivity of compounds bound to the estrogen receptors. Among them propylpyrazole triol (PPT) and methyl piperidinopyrazole (MPP) were found ER$\alpha$ selective, whereas DPN, Genistein and

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Daidzein were ERβ selective. The Relative Binding Affinities (the EC50 ratio between ERα and ERβ) of those compounds were in correlation with the literature; PPT (593), MPP (220), DPN (0.01), Genistein (0.002) and Daidzein (0.2) (Skretas & Wood, 2005b). Additionally, the TR biosensors were able to detect Triac, GC-1 and KB-141 TRβ selectivity (unpublished results), which are also in agreement with reported results (Bleicher et al, 2008; Grover et al, 2005; Koury et al, 2009; Li et al, 2006; Marimuthu et al, 2002; Martinez et al, 2009; Scanlan, 2008; Wagner et al, 2001).

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<tr>
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Table 1. Summary of the NHR bacterial biosensor assays and their conditions, including temperatures and growth medium additives for optimizing results (Skretas et al, 2007). The +/- signs indicate cell growth below or above the baseline OD600 value obtained for cells in the presence of solvent only (which is constant throughout the sample and not higher than 1%). Abbreviations: TTM: -THY medium with 50µg/mL Thymine and 10µg/mL Trimethoprim; +THY: -THY medium with 50µg/mL Thymine; E2 is 17-β-estradiol, typically at 0.1 to 10 µM depending on the test strain.

2.2.4 Effect of compounds across different species
We also confirmed in our studies that the effects of tested compounds varied across different species, such as sole (Solea solea) and human (Gierach et al, 2011). As expected, compounds expressed similar effects when bound to pig and human ERβs, but in some cases diverged for sole and human or pig. The fact that the biosensors are able to quantify differential effects across species can allow rapid screening of ED pollutants using consistent assay protocols, which will eliminate important barriers to comparing these types of data in the current literature. This creates great opportunities for understanding crosstalk of receptors in a wide range of species, and allows the selectivity of ligands across receptors and species to be explored.

2.2.5 Synergism and competition of natural hormones, pharmaceuticals and EDCs
There is a great concern that mixtures of EDCs could have stronger and more devastating effects than single compounds on health and environment. Since there is no limitation as to how many ligands can be tested simultaneously, the biosensor could detect synergistic effects acting through the NHR LBD as well.

3. Bacterial biosensors in drug discovery
The process of delivering new pharmaceuticals to the market takes approximately 10-15 years and only one compound in 10,000 has a chance to be approved by the U.S. FDA. The
development cost per drug can reach $800 million on average (Brower, 2002). For these reasons, there is an urgency to discover new compounds that are more selective and have fewer side effects. Early stage discovery and pre-clinical research may take 6-7 years alone.

Fig. 7. The process of designing a bacterial biosensor and its utilization. First, the sequence of the plasmid is constructed computationally. The template (e.g., pMIT::ER_α) stays intact, and the insert (N-terminal intein-NHR LBD-C-terminal intein) is swapped to create a new biosensor with a different LBD. Next, the plasmid is transformed into a bacterial cell, which allows the encoded gene to be transcribed and translated into the active biosensor protein. In the presence of an agonist, the activity level of the TS reporter enzyme domain is increased, leading to an increase in cellular growth rate (see the graph above). However, when antagonists are present the activity of the TS is low, which allows a distinction between these two types of the compounds. Cell growth can be quantified by OD_600 measurements. The 3D structures of NHR LBDs proteins (such as the ER LBD shown above) can be found in the RCSB Protein Data Bank (PDB).

Therefore, the development of new methods to rapidly screen millions of ligands against new targets is essential and ongoing. The identification of ligands that bind to estrogen receptor(s) began with the development of treatments for patients with hormonal dysfunctions, cancers, and sexually transmitted bacterial infections. Several early pharmaceuticals, such as diethylstilbestrol (DES, a nonsteroidal estrogen synthesized just before World War II by Leon Golberg), failed due to their high toxicity and disastrous side effects, such as breast cancer and vaginal adenocarcinoma in second-generation girls (Birch, 1992; Jordan et al, 2008). The first human ER was discovered in 1966 by Jensen and Gorski, and it took an additional 30 years until the first mammalian ER_β sequence was cloned (Fannon et al, 2001). Interestingly, the compound tamoxifen, as well as second-generation benzothiophene derivative selective estrogen receptor modulators (SERMs) (e.g., raloxifene, known as Evista® (Pritchard, 2001; Wilkinson et al, 1982)), show unique selective action in targeted tissues. In some tissues SERMs act as an estrogen, and therefore their action is described as agonistic, but in others they block the effect of estrogen and behave as antagonists. In many cases, the mechanism of SERM action is not fully understood, and research is ongoing on the roles of the co-regulators, ligands, and cross-signaling proteins that mediate ER expression levels across the human body. For example, it was determined that ER_β is dominant in the gastrointestinal tract, whereas ER_α dominates in liver. Both of these receptors are expressed in breast, bone and brain tissue, but in different ratios,
allowing direct targeting of specific organs (Gustafsson, 1999). During the last 10 years, the number of targeted drug-like compounds produced in industry and academia has increased dramatically. These compounds have been developed as fertility drugs, as well as breast cancer, prostate cancer and osteoporosis therapeutics. Among them are Toremifene (Fareston® by Shire laboratories), lasofoxifene, troxifene, droloxifene, clomifene (Clomid® by Hoechst Marion Roussel, Inc.) and ormeloxifene (originally manufactured by Torrent Pharmaceuticals), which was used as a birth control pill, and is now also prescribed to cure uterine bleeding (Blizzard, 2008; Fan et al, 2007; Musa et al, 2007; Sanceau et al, 2007). Pure antagonists were also synthesized such as ICI 182,780 (Faslodex® by AstraZeneca), which is known as a selective estrogen receptor downregulator; SERD (Abdou et al, 2008).

In previous research, we showed that bacterial biosensors could detect novel compounds and determine their behavior as agonists or antagonists (see structures of discovered compounds in Fig. 8 and Table 2) (Hartman et al, 2009; Skretas et al, 2007). Compounds a and b were found to bind to ER, and their agonistic and antagonistic effects were verified using biosensors. The new findings were confirmed by a fluorescence polarization displacement assay using extracts of human ERβ and ERα and fluorescently labeled estrogen, as well as by analyzing ERE-dependent luciferase gene activity in human embryonic kidney HEK:ERβ and breast cancer MCF-7: D5L cells. The relative binding affinities of these compounds, determined by competitive binding assays, showed the ERβ selectivity of compound b. Specifically, the RBA (relative binding affinities of the compound to estrogen) of compound a and b for ERα were 0.23±0.03 and 0.59±0.09, and for ERβ were 1.94±0.024 and 0.78±0.10, respectively (Skretas et al, 2007). An additional study using a luciferase reporter system revealed that compound a is an agonist, but compound b is a partial agonist and partial agonist/antagonist when bound to ERα and ERβ, respectively.

Table 2. Bacterial biosensor results obtained for hormones and pharmaceuticals screened against human ERβ (Skretas et al, 2007). Concentration of the ligands was 5 µM, with the exception of tamoxifen at 2 µM. Antagonists were tested in competitive assays against 500 nM E2. The (+/-) symbols indicate a positive/negative cell growth outcome.

<table>
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<tr>
<th>Compound</th>
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<td>Estrone</td>
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movie industry to computationally draw shapes of objects in 3D space. The solvent-accessible surface of each molecule is defined by a Smooth Molecular Surface Triangulator algorithm (Zauhar, 1995), and is essential for defining the volume of a molecule. The shape of the molecule, as well as the molecular electrostatic potential computed over the surface of the molecule, can be compared across large databases and scored rapidly for the most promising compounds. The two molecules c and d (Fig. 8) were derived from a Shape Signatures screening against GW7604 and EM652 (see Fig. 3).

Compounds c and d were determined to bind to the active pocket of ER using Computer-Aided Drug Design methods, and their behavior was examined using the bacterial biosensors and an ERα activation immunoassay (ELISA) containing MCF-7 cell extract (Hartman et al, 2009; Skretas et al, 2007). The predicted binding mode of compound c overlapped the position of Tamoxifen, which binds through weaker van der Waals interactions to the active pocket. Compound d showed possible hydrogen bond formation with Glu353. Overall, compounds c and d were found to be orally bioavailable by Lipinski’s Rule of Five, which takes into account solubility, molecular weight, and even the number of rotational bonds and hydrogen bond donors and acceptors (Hartman et al, 2009).

![Fig. 8. The structures of agonists and antagonists discovered and confirmed by commercial as well as conventional methods ER (Hartman et al, 2009; Skretas et al, 2007). Names of the compounds: (a) 5-[(1E,3E)-4-(4-hydroxyphenyl)-1,3-butadien-1-yl]-1,3-benzenediol; (b) 2(1,1-dimethylethyl)-4-[(1E)-2-(4-hydroxyphenyl)ethenyl]-phenol; (c) 3-(2aminophenyl)sulfanyl-1-(4-chlorophenyl)-3-phenyl-propan-1-one; (d) 3-(2aminophenyl)sulfanyl-3-(4-bromophenyl)-1-phenyl-propan-1-one.]

4. Environmental endocrine disruptors detection

Synthetic or natural compounds that interfere with hormonal and homeostatic systems are known as Endocrine Disruptors (EDs) (Nilsson, 2000). The targets of EDs can be NHRs, non-hormonal receptors, or numerous biological pathways involved in hormone synthesis, metabolism and excretion. Thus, EDs can cause serious disruption of the endocrine system by mimicking natural hormones, which may lead to increased or inhibited transcription of hormone-regulated genes. Some of these compounds are now associated with various cancers, genetic and reproductive diseases, as well as behavioral and developmental abnormalities. A few ED-associated disorders seen in humans are also manifest in other species, and include uterine leiomyomas, endometriosis, cancers, diabetes and obesity (Bryzgalova et al, 2008; Cook et al, 2007; Goksoyr, 2006; Koda et al, 2007; Kuiper et al, 2007;
Lee et al, 2007; Lingxia et al, 2007; Maffini et al, 2006; Negri-Cesi et al, 2008; Nilsson, 2000; Safe, 2000). EDs comprise a wide range of chemicals, including solvents, pesticides, and pharmaceuticals (e.g., DES), and even naturally occurring phytoestrogens in plants (McKinlay et al, 2008). Some EDs accumulate in the body, such as uranium from nuclear power plants, lead, mercury and cadmium, which travel to the brain and kidney (Raymond-Whish et al, 2007; Strumylaite et al, 2008; Vahter et al, 2007). Some weakly binding compounds, such as BPA, are embedded in plastic bottles used for water and soft drinks (Vandenberg et al, 2007). In particular, BPA has been shown to bind to hormone receptors across different species. Despite numerous studies on the negative effects of BPA (Vandenberg et al, 2009), to this day its impact on the body is still controversial. However, the EPA and FDA are now moving to update their disclosures on BPA in response to an internal BPA study showing negative effects of exposure on the brain and prostate, as well as on the behavior of infants and children (Keri et al, 2007).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexestrol</td>
<td>Synthetic, DES derivative</td>
</tr>
<tr>
<td>Genistein</td>
<td>Isoflavone, found in plants e.g. soybeans</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>Flavonoid, found in plants e.g. tea, apples</td>
</tr>
<tr>
<td>Naringenin</td>
<td>Flavonoid, found in plants e.g. grapefruit</td>
</tr>
<tr>
<td>Diphenylnitrosamine (DPN)</td>
<td>Synthetic</td>
</tr>
<tr>
<td>BPA</td>
<td>Plastics; e.g. bottles, caps</td>
</tr>
<tr>
<td>Apigenin</td>
<td>Flavonoid, found in plants e.g. parsley, celery</td>
</tr>
<tr>
<td>Zearalanols</td>
<td>Found in plants e.g. fungi (Fusarium)</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>Flavonoid, found in plants e.g. peanuts, soy</td>
</tr>
<tr>
<td>Daidzein</td>
<td>Isoflavone, naturally found in plants e.g. soybeans</td>
</tr>
<tr>
<td>Phloretin</td>
<td>Found in plants e.g. apple leaves</td>
</tr>
<tr>
<td>Naringenin</td>
<td>Flavonoid, found in plants e.g. grapefruit</td>
</tr>
</tbody>
</table>

Table 3. Selected ER agonists detected by bacterial biosensors, including phytoestrogens and synthetic estrogen analogs. BPA, DPN and 17-β-estradiol (natural hormone, ER agonist) were additionally confirmed to be agonists in the sole and pig ERβ biosensors. BPA and Daidzein, despite the fact that they are weak ER agonists, were also detected (Breinholt & Larsen, 1998; Collins et al, 1997; Kuiper et al, 1997; Kuiper et al, 1998; Skretas et al, 2007).

One of the difficulties in determining the impact of EDs on the human body is that although a broad range of assays are available, their results are generally not directly comparable. For example, the detection limit of a given compound can vary by a factor of 1000 or more across several different in-vivo and in-vitro assays (Charles, 2004; Dobbins et al, 2008; Kramer, 1998). Measuring the impacts of these compounds on humans is also very difficult, especially when evaluating the cumulative impact of EDs over time on the body. Often, these assays cannot distinguish between high overall toxicity of EDs and their agonistic and antagonistic effects. Non-sigmoidal functions are also observed for some EDs, which exhibit U-shaped and inverted-U-shaped dual dose response curves, such as those seen for phytoestrogens and neurotransmitters. For example, at low concentrations some compounds may act as agonists, but at high concentrations they may behave more like
antagonists (Li et al, 2007; Pinto et al, 2008). Additionally, EDs typically exist in the environment in mixtures, and therefore there is a need for newly developed HTS assays to assess their effects alone and in combination with other compounds (Charles et al, 2007). The effect of an ED alone or in a mixture of EDs in different assays may also vary (Benachour et al, 2007; Ramamoorthy et al, 1997). Since EDs can be accumulated in the body, their half-life could be longer than expected, and contact with these chemicals more frequent than expected. For example, the BPA daily safe uptake is 50 µg/kg as determined by the EPA, but it accumulates in fat tissue over time. Therefore, additive daily exposure occurs, resulting from a longer than expected half-life in the human body (Diamanti-Kandarakis, 2009). Therefore, understanding the impact of these chemicals on the endocrine system and human health is challenging, especially for weakly binding EDs like BPA. The bacterial biosensors can easily differentiate between bacterial toxicity and endocrine activity. Examples of compounds that bind to ERs, and their sources, are shown in Table 3. Some of them are naturally occurring chemicals found in plants, but others are synthetic. In each case, these compounds mimic estrogen, but their structure is not typically steroid-like (see examples of DES, BPA in Fig. 3). In some cases, the compounds could be beneficial (e.g., chemopreventive properties of Apigenin; antidepressant properties of Kaempferol) as well as harmful or epigenetic (BPA, DES and Hexestrol).

4.1 Screening of home products for estrogenic activity

The intein-based biosensors are capable of detecting small amounts of estrogenic compounds in consumer products such as perfumes, pills and plant extracts. A study using the bacterial biosensors showed that chemicals such as benzophenone-2 (see Fig. 3), which is a UV absorber used in plastic food containers and cosmetics, can be detected by this method. Benzophenone-2 (BP-2) has an agonistic effect on the human ER, and several publications suggest estrogenic effects on fish (juvenile fathead minnows) as well rats (Kunz et al, 2006; Schlecht et al, 2008; Seidlova-Wuttke et al, 2004). In other studies, BP-2 was shown to have an agonistic effect on fish (rainbow trout) and human ERs, and was found to be selective for human ERβ in an in vitro bioassay (Molina-Molina et al, 2008). BP-2 was also tested in pregnant mice to determine its impact on fetuses, where it was found that BP-2 may be a cause of hypospadia which is an abnormality in the reproductive organs of male fetuses (Hsieh et al, 2007). Additionally, BP-2 suppresses T4 (thyroid hormone), but not T3, and has estrogenic activity in rats (Seidlova-Wuttke et al, 2005). The results obtained by the bacterial biosensors also suggest an agonistic effect of BP-2 on the human ERβ. Several perfumes were tested, including Notes by Celine Dion, Amber Romance by Victoria’s Secret, Roma by Laura Biagiotti and Haiku by Avon. All of the tested perfumes that contain BP-2 as an ingredient had an agonistic effect on ERβ biosensor, but no effect on the TRβ biosensor. Tests of several natural menopause-relief pills showed a weak positive effect in many cases as well, which included Black Cohosh and Dong Quai Capsules by Nature’s Way, and Black Cohosh and Dong Quai Capsules by Gaia Herbs. Notably, essential oils (e.g., Lavender Oil by Plantlife) and soaps were screened but were toxic to the bacterial cells (Gawrys et al, 2009).

4.2 Testing endocrine disruptors across species

The human NHR LBDs of the biosensors can be easily exchanged for animal LBDs and the effects due to the same set of ligands can be compared. The sequences of ERβ for Sus scrofa...
and *Solea solea* were inserted in place of human ERβ in the pMIT::ERβ*, and the potencies of DES, BPA, DPN, Daidzein and E₂ were compared. The potencies across species showed that DES and E₂ have the strongest effect on all of fish, pig and human biosensors. DES was almost twice as effective on pig than on fish or human. The weakest effect was noticed for BPA in the order human > fish > pig. The half maximal effective concentrations (EC₅₀) were compared to determine relative pseudotransactivation (RPTA, see Equation (1)). The EC₅₀ values and the standard deviations of data obtained in triplicate can be presented as sigmoidal plots of OD vs. log of test compound concentration. The calculations were based on nonlinear regression with variable Hill slope (GraphPad Prism 5.01; GraphPad Software, La Jolla, CA, USA).

\[
\text{RPTA} = \frac{\text{EC}_{50}^{\text{ligand}}}{\text{EC}_{50}^{\text{E2}}} \times 100\% 
\]

(1)

However, the RPTA values for DPN were quite close to E₂ for human (~80%), but showed it to be less potent for pig (33%) and fish (12%). An even smaller relative effect was seen for Daidzein, commonly found in soybeans, on human ERβ (4%) = pig (4%) > fish (0.9%). Similarly, BPA had very little effect on fish (5.6%) > human (0.9%) > pig (0.23%). The two-tailed p values with 95% confidence interval verified significant correlation between EC₅₀ values of tested compounds on human and porcine ERβs. There was no correlation of those ERs with sole ERβ. To further examine the quality of the assay, the Z' factor for each measurement was calculated, which is an indication of signal to noise in a measurement (see Equation (2); (Zhang et al, 1999)). In both cases the data were sufficient to distinguish ER agonists across species in HTS set up, where Z' factor above zero is needed to robustly determine whether a given ligand is a potential EDC. In general, all of the tests produced very good Z' factors, ranging from the strong agonist E₂ (Z' factor of 0.4-0.6 across the three tested species), to the weaker agonist BPA (Z' factor of 0.15-0.32) (Gierach et al, 2011).

\[
Z' = 1 - \frac{3x(\text{SD}_{\text{max}} + \text{SD}_{\text{min}})}{\overline{\text{Mean}}_{\text{max}} - \overline{\text{Mean}}_{\text{min}}}
\]

(2)

The data obtained here were also compared to the literature. The trends of recorded effects of ligands on human and pig ERs were similar (DES >> E₂ >> Daidzein > BPA). Weaker binders like Daidzein and BPA were also tested previously in a yeast transcriptional assay, and the values obtained from these studies closely correlated with our findings (2.5x10⁻⁷ M (yeast assay) vs. 1.7x10⁻⁶ M (bacterial assay) and 1.45x10⁻⁷ M (yeast assay) vs. 3.6x10⁻⁷ M (bacterial assay), respectively; (Chu et al, 2009)). The performance of the *Solea solea* biosensor was more difficult to ascertain, due to a relative lack of available data compared to carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) (Matthews et al, 2000; Matthews et al, 2001; Petit et al, 1995). In general, however, in *vivo* tests in other piscine species were less sensitive for weak binders, such as BPA. In this case, a hepatocyte vitellogenin secretion test at up to 100 µM concentration of BPA showed limited response (Smeets et al, 1999). Our studies were able to determine the binding effect of BPA in sole at lower concentrations (0.10 to 9.85 µM). In other previous assays where piscine ERs were tested, trends in the strength of binding were similar to those observed for Daidzein and DPN in the bacterial biosensors. The alternate assays included a displacement assay using a trout ER nuclear extract, or the previously mentioned hepatocyte vitellogenin secretion assay. A correlation
between all of these assays was seen for strong binders, such as $E_2$, which closely corresponded with in vivo studies: 50-150 nM (vitellogenin assay) (Smeets et al, 1999) vs. 21-153 nM (bacterial biosensor assay).

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection</th>
<th>Bacterial Host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heavy metals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green fluorescent protein (GFP)-based bacterial biosensors</td>
<td>Cd(II), Pb(II), and Sb(III) in sediments and soils</td>
<td><em>Escherichia coli</em></td>
<td>(Liao et al, 2006)</td>
</tr>
<tr>
<td>GFP bacterial biosensor</td>
<td>As</td>
<td><em>Escherichia coli</em></td>
<td>(Tani et al, 2009)</td>
</tr>
<tr>
<td>Bioluminescent bacterial biosensor</td>
<td>Cu, Zn, Cd, Co, Ni, Pb</td>
<td><em>Alcaligenes eutrophus</em></td>
<td>(Collard et al, 1994; Diels et al, 1999)</td>
</tr>
<tr>
<td>Fibre-optic luminescent bacterial biosensors</td>
<td>Hg and As in soils and sediments</td>
<td><em>Escherichia coli</em></td>
<td>(Ivask et al, 2007)</td>
</tr>
<tr>
<td><strong>Antibiotics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorimetric bacterial biosensor dipstick-based technology</td>
<td>Tetracycline, streptomycin and macrolide in food</td>
<td><em>Escherichia coli</em></td>
<td>(Link et al, 2007)</td>
</tr>
<tr>
<td>Luminescent bacterial biosensor</td>
<td>Tetracyclines in poultry muscle</td>
<td><em>Escherichia coli</em></td>
<td>(Pikkemaat et al, 2010)</td>
</tr>
<tr>
<td><strong>Hormones, Pharmaceuticals, Endocrine Disruptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell growth based TS-deficient NHR bacterial biosensors</td>
<td>Wide variety of compounds e.g. estradiol, $T_3$, triac, tamoxifen, GC-1, diethylstibestrol, KB-141, daidzein, DPN and genistein</td>
<td><em>Escherichia coli</em></td>
<td>(Gawrys et al, 2009; Hartman et al, 2009; Skretas et al, 2007; Skretas &amp; Wood, 2005a, 2005b, 2005c)</td>
</tr>
<tr>
<td>Electrochemical bacterial biosensors</td>
<td>Aromatic hydrocarbons and heavy metals</td>
<td><em>Escherichia coli</em></td>
<td>(Paitan et al, 2003)</td>
</tr>
<tr>
<td>Fluorescent and luminescent toluene bacterial biosensors</td>
<td>Environmental pollution with petroleum products e.g. benzene, toluene, ethylbenzene, and xylenes</td>
<td><em>Escherichia coli</em></td>
<td>(Li et al, 2008)</td>
</tr>
<tr>
<td>Bioluminescent naphthalene biosensor</td>
<td>Naphthalene</td>
<td><em>Pseudomonas putida</em></td>
<td>(Werlen et al, 2004)</td>
</tr>
<tr>
<td><strong>DNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bioluminescent bacterial biosensor for DNA damage, alkylation and mutagenicity recognition</td>
<td>Genotoxicants included: endocrine disrupting chemicals, phenolitics and compounds causing oxidative stress (e.g. $H_2O_2$, CdCl)</td>
<td><em>Escherichia coli</em></td>
<td>(Ahn et al, 2009)</td>
</tr>
<tr>
<td>Microgravity and space radiation bacterial biosensors</td>
<td>Analysis of the level of radiation exposure on human body by bacterial detection</td>
<td><em>Salmonella typhimurium</em></td>
<td>(Rabbow et al, 2003)</td>
</tr>
<tr>
<td>Stress-responsive bacterial biosensors</td>
<td>DNA damage by oxidative and genotoxic conditions</td>
<td><em>Escherichia coli</em></td>
<td>(Mitchell &amp; Gu, 2004)</td>
</tr>
</tbody>
</table>

Table 4. The review of bacterial biosensors usage.
5. Brief overview of other bacterial biosensors

The intein-NHR bacterial biosensors are useful for ED screening and drug discovery, and exhibit many advantages over conventional assays. These advantages are also observed in other bacterial biosensor systems, which have been extended into wide range of applications. These include testing for antibiotics in food, as well as detecting DNA damage by chemicals and even space radiation. Several examples of other bacterial biosensors are included in Table 4.

6. Conclusions

The bacterial biosensors presented here are an excellent tool for screening EDs, pharmaceuticals, hormones or mixtures of compounds for their agonism or antagonism against human NHRs as well as NHRs of other species. These biosensors meet the need for a method to rapidly compare the effects of NHR ligands across different species, and to estimate the potential danger of chemicals in the environment. These bacterial biosensors can be also used to rapidly and cheaply test large amounts of the unknown chemicals for possible future uses as lead compounds in pharmaceutical research, including compounds with receptor sub-type selectivity. The simplicity of the assay and very low cost are attractive key features of this method.

7. Acknowledgment

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8. References


A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 16 different countries. The book consists of 24 chapters written by 76 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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