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Tamoxifen-Mediated Apoptosis in ER Negative Breast
Cancers

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13. ABSTRACT (Maximum 200 Words) Estrogens are small-molecule hormones that control the growth of ~ 50 % of all breast cancers. Tumors that require estrogens for growth are termed estrogen receptor positive (ER+) because they express estrogen receptors (ERs) that regulate cellular proliferation. Other breast cancers are termed ER negative (ER-) and do not require estrogens for growth. One pronounced difference between ER+ and ER- breast cancers regards the expression of the enzyme estrogen sulfotransferase (EST), which metabolizes estrogens and antiestrogen chemotherapeutics to form inactive sulfates. Since EST inactivates estrogens, ER+ breast cancers generally lack EST activity, whereas ER-negative (ER-) breast cancers often exhibit elevated levels of EST activity. Our laboratory is developing novel methods to study the effects of small molecules on ERs and EST.				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusions.....	5
References.....	5
Appendices.....	5

Introduction

Small molecule – protein interactions underlie many fundamental processes in biology and provide a basis for pharmacological intervention in human disease. Given that most proteins are only marginally stable, molecular recognition between small molecules and enzyme active sites or protein ligand binding domains (LBDs) often stabilizes protein folding. Ligand-mediated protein stabilization has been recently employed in yeast assays to couple cellular growth to ligand binding of proteins fused to the essential metabolic enzyme dihydrofolate reductase.¹ The autocatalytic green fluorescent protein (GFP) from *Aequorea victoria*² has also been utilized as a folding reporter that confers a fluorescent signal proportional to the extent of folding of fused proteins.³ Proteins have also been inserted into loops on the surface of GFP to construct biosensors.⁴ Most cellular biosensors of small molecules are based on relatively complex two-hybrid systems, in which ligand binding is used to trigger protein dimerization to activate expression of a reporter gene.⁵ However, ideal biosensors would be homogenous (reagentless) and provide a reporter function intrinsic to the sensor molecule without requiring covalent modification or assembly of macromolecular components.

Body

We report herein a novel and simple method to detect interactions between therapeutically important steroid hormone receptor LBDs and small molecule ligands such as compounds 1-9. As shown in Figure 1, our method involves coupling the fluorescence of the red-shifted mutant of GFP termed yellow fluorescent protein (YFP)⁶ to ligand binding-induced protein stabilization or destabilization of steroid hormone receptor LBDs expressed in living yeast cells. Fusion proteins were designed from X-ray crystal structures of YFP (PDB# 1YFP)⁶ and ligand-bound estrogen receptor- α (ER α , PDB# 3ERT),⁷ estrogen receptor- β (ER β , PDB# 1HJ1),⁸ and androgen receptor (AR, PDB# 1I37)⁹ proteins. These fusion proteins directly link the N-terminus of YFP to the C-terminus of the steroid receptor LBD via a short Val-Glu dipeptide to closely couple effects on receptor folding to the fluorescence of YFP (Figure 2).

To assess the effectiveness of intracellular YFP as a sensor of folding of LBD fusion proteins, temperature, temporal, and ligand-dependent fluorescence of living recombinant yeast cells was quantified by flow cytometry. As shown in Figure 3A, the fluorescence of intracellular YFP was highly dependent on the cellular growth temperature. High fluorescence values were obtained at the suboptimal growth temperature of 20 °C, but lower values were measured at yeast growth temperatures of 30 °C or 37 °C, indicating that at 20 °C folding of the fluorescent fusion protein is favored, enhancing YFP fluorescence, but higher temperatures destabilize the protein fold and reduce fluorescence.

To investigate ligand-induced effects on cellular fluorescence, ligands 1, 4, and 7, bearing high affinity for ER α (1: $K_d \sim 0.3$ nM),¹⁰ ER β (1: $K_d \sim 0.9$ nM),¹⁰ AR (4: $K_d \sim 0.5$ nM),¹¹ and glucocorticoid receptor (GR, 7: $K_d \sim 1.4$ nM),¹² were added to recombinant yeast that were grown at 30 °C and resuspended in sterile water to stop cellular growth. Subsequent addition of cognate ligands substantially enhanced cellular LBD-YFP fluorescence with maximal values obtained ca. 6 h after compound addition (Figure 3B). The short linker between the ER proteins and YFP was critical for this effect (data not shown). That non-dividing cells exhibited ligand-mediated fluorescence enhancements suggests that these ligands promote folding of a pre-existing population of poorly folded intracellular protein to produce a functional fluorescent product.

To examine the specificity of ligands for LBD-YFP proteins, recombinant yeast were treated with ligands 1-9 and effects of all of these compounds on the fluorescence of each receptor was quantified by flow cytometry. These effects were highly correlated with conventional transcriptional yeast two-hybrid assays that measure ligand-induced dimerization of steroid hormone receptor LBDs (Figure 4). Furthermore, cognate ligands conferred dose dependent fluorescence enhancements that paralleled literature values of relative receptor binding affinities *in vitro*.

Remarkably, in some cases the cellular fluorescent folding sensors were more sensitive than analogous two-hybrid assays. For example, GR ligands 6, 8, and 9 provided significant 4 to 5-fold fluorescence enhancements when tested against GR-YFP, but these ligands were not detected (< 2-fold effects) with the corresponding transcriptional assay. Furthermore, these sensors can discriminate between highly structurally similar molecules such as detection of the hydroxyl group of testosterone (5) but not the analogous methyl ketone of progesterone (8) (Figure 4C).

In addition to stabilizing ligands, destabilizing ligands can also be identified by growing recombinant yeast at 20 °C to favor protein folding. This approach identified progesterone (8) as a weakly destabilizing ligand of ER α and ER β fusion proteins (Figure 5). Although the physiological significance of this effect of progesterone remains to be established, based on this observation, we postulate that the identification of compounds that destabilize protein folding could lead to the discovery of novel classes of receptor antagonists. The approach described herein provides a potentially general method for analysis of diverse small molecule – protein interactions.

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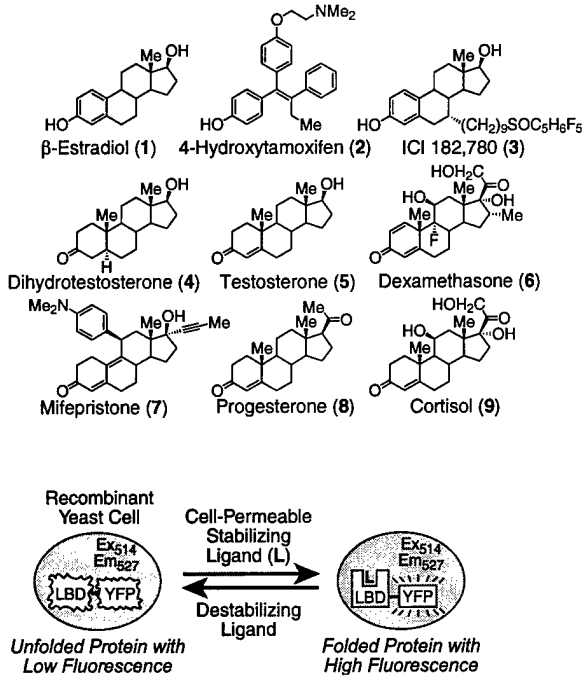


Figure 1. Design of a fluorescent cellular sensor that detects ligand-mediated stabilization or destabilization of receptor LBDs.

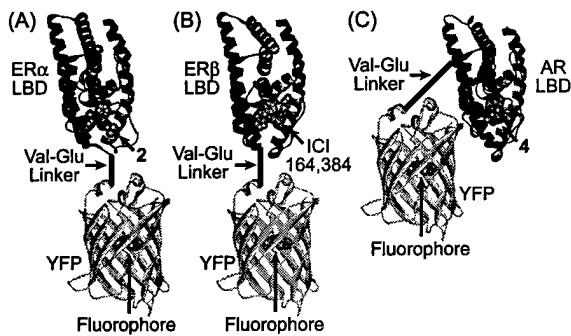


Figure 2. Models of steroid hormone receptor-YFP fusion proteins. (A) ER α LBD-YFP. (B) ER β LBD-YFP. (C) AR LBD-YFP.

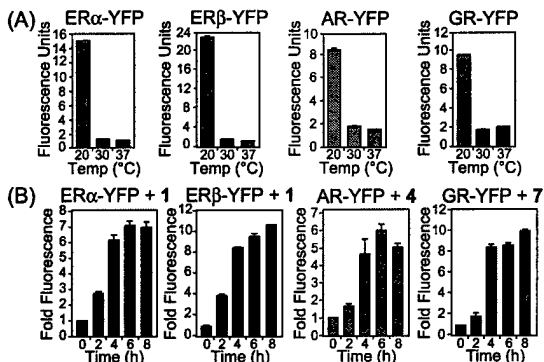


Figure 3. Whole cell fluorescence measured by flow cytometry. (A) Modulation of cellular growth temperature. (B) Treatment of non-dividing living cells with ligands (10 μ M) for the times indicated.

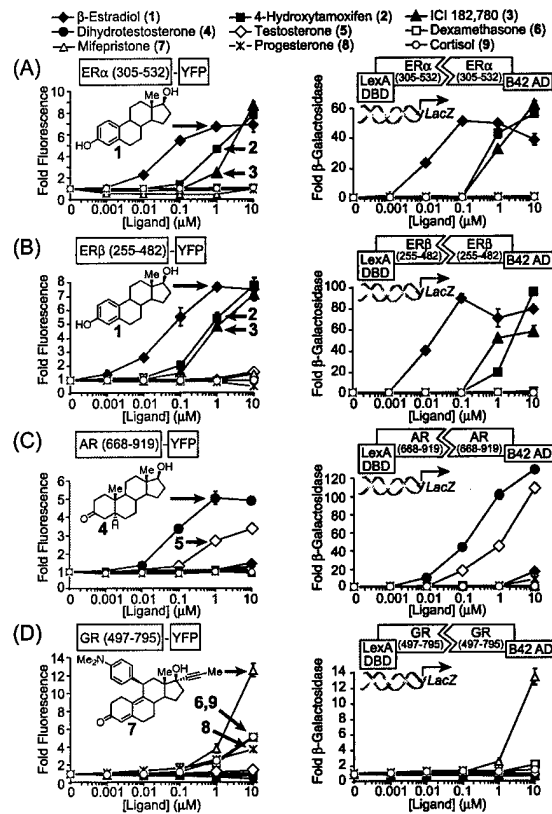


Figure 4. Comparisons of whole cell dose-response curves from steroid receptor LBD-YFP fluorescence measured by flow cytometry (left panels) and conventional transcriptional two-hybrid assays (right panels). Structures of the most potent ligands are shown. In the two-hybrid assays, the LBDs are expressed as two fusion proteins: a LexA DNA binding domain (DBD) fusion and a B42 activation domain (AD) fusion. Ligand-induced dimerization of the LBDs activates transcription of a *LacZ* reporter gene by reconstituting a functional transcriptional activator. (A) ER α assays. (B) ER β assays. (C) AR assays. (D) GR assays.

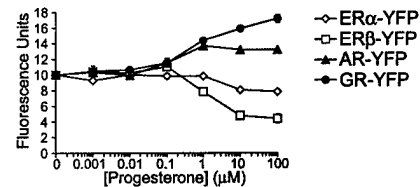


Figure 5. Effects of progesterone on fluorescence of LBD-YFP proteins in yeast grown at 20 $^{\circ}$ C. Basal fluorescence values were normalized to ten units to facilitate the comparison.

Key Research Accomplishments

Key research accomplishments include the development of novel methods to identify small molecules that influence the folding of estrogen receptor proteins. These methods may enable the identification of novel anti-breast cancer agents.

Reportable Outcomes

This Concept Award, in addition to funds provided by an American Cancer Society Research Scholar Grant For Beginning Investigators, funded a study that was submitted for publication to *J. Am. Chem. Soc.*

Conclusions

This concept award successfully funded the generation of data that was submitted for publication.

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Appendices - None