Receptor Ligands

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Monitoring Ligand-Induced Conformational Changes for the Identification of Estrogen Receptor Agonists and Antagonists**

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Abstract: *Nuclear receptors are transcription factors that are important targets for current drug discovery efforts as they play a role in many pathological processes. Their activity can be regulated by small molecules like hormones and drugs that can have agonistic or antagonistic functions. These ligands bind to the receptor and account for diverse conformational changes that are crucial determinants for the receptor activity. Here, we set out to develop FLiN (fluorescent labels in nuclear receptors), a direct binding assay that detects conformational changes in the estrogen receptor. The assay is based on the introduction of a cysteine residue and subsequent specific labeling of the receptor with a thiol-reactive fluorophore. Changes in the receptor conformation upon ligand binding lead to differences in the microenvironment of the fluorophore and alter its emission spectrum. The FLiN assay distinguishes between different binding modes and is suitable for high-throughput screening.*

The estrogen receptor (ER) belongs to the protein superfamily of nuclear receptors (NRs), which has 48 members. These ligand-dependent transcription factors play a fundamental role in physiological functions such as control of cell differentiation, homeostasis, organ physiology, and embryonic development.[1] Moreover, they are involved in pathological processes, such as diabetes, cancer, asthma, rheumatoid arthritis, and hormone resistance syndromes.[2] These findings have turned NRs into favored targets for pharmaceutical intervention and have resulted in the development of a variety of small-molecule regulators that are currently used to treat several human diseases. The ER displays a modular structure, characteristic for all NRs, comprising an N-terminal activation function, a central DNA-binding domain, and a hinge region that forms the connection to the C-terminal ligand-binding domain (LBD).[3] The latter mediates ligand binding, receptor dimerization, and nuclear localization. Lipophilic small-molecule ligands can bind to the LBD and induce distinct conformational changes in the C-terminal helix 12, which modulate the ER activity for transcription of the target genes.[4] These ligands play a critical role for receptor activity. Depending on the conformation of helix 12, different co-regulator proteins can be recruited and the ligands can act purely as agonistic agents such as the natural hormone 17β-estradiol (E2) and as selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene. These agents display tissue selective agonistic or antagonistic effects.[5] The receptor–ligand interactions have been widely studied using various methods like NMR spectroscopy,[6] co-regulator interaction assays,[7] and X-ray crystallography.[8] It is now becoming clear that individual receptor–ligand complexes promote unique conformations that exhibit different affinities for the various co-activators and co-repressors, thereby controlling the expression of distinctive sets of genes. Deciphering the relationship between conformational changes and ligand structure is essential for the understanding of the mechanistic details and the selective pharmacological profiles of certain ligands. Despite decades of research in the field, the identification of new NR ligands is still of great interest for biomedical investigation and drug discovery.[9] The detection of selective ligands holds promise for the development of highly effective drugs with improved therapeutic profiles, while minimizing unwanted side effects. In this respect, high-throughput direct binding assay systems that monitor conformational changes in the LBD and allow for rapid and cost-efficient identification of new ligands are of major interest for medicinal chemistry and chemical biology research.

Herein, we describe a fluorescence-based direct binding assay for the identification of agonists and antagonists of the human ERβ, one of the two ER isoforms that are encoded on different genes. To date, ERα is the main therapeutic target, but ERβ is evolving to a promising target for the development of innovative drugs. In particular receptor-subtype-selective ERβ agonists have the potential for the development of new therapies with reduced side effects.[10]

The assay system is based on the detection of conformational changes in helix 12 upon the binding of the ligand to the LBD (Figure 1). The orientation of the C-terminal helix 12 in the LBD is a main determinant for agonistic and antagonistic effects of the ligand. In an active, agonist-bound state, helix 12 is bent by approximately 90°, creating a binding site for co-activators. In the inactive antagonist-bound state, helix 12 occupies the co-activator site and prevents co-activator binding. However, in most cases, this static picture produced by crystallographic studies is insufficient to explain the complex outcomes of ligand-binding to NRs.

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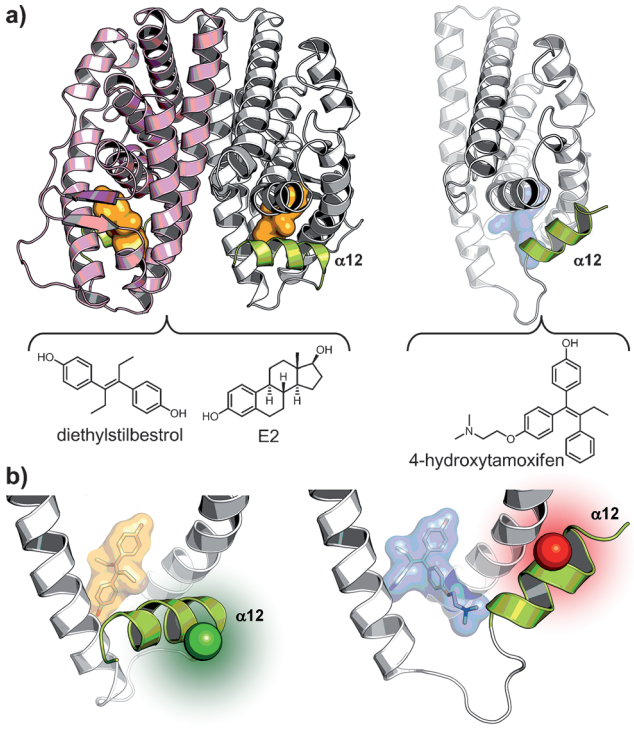

Figure 1. Principle behind the FLiN assay for the detection of ER LBD ligands. a) Ligand binding (orange and blue) induces different conformations of helix 12 (highlighted in green) in the ER LBD (PDB entries 1ERD and 1ERT). Crystal structures from ERα are shown, as no structures of human ERβ with these ligands have been reported. The two LBDs share a high structural identity and the binding of agonists and antagonists to ERβ induces similar conformations.[11] b) A cysteine residue was introduced into helix 12 by site-directed mutagenesis and labeled with the environmentally sensitive fluorophore acrylodan (green and red spheres). Conformational changes of helix 12, upon binding of ER agonists and antagonists, lead to alterations in the microenvironment of the fluorophore, thereby altering its fluorescence properties.

The direct binding assay relies on changes in the fluorescence spectrum of an environmentally sensitive fluorophore that senses conformational changes in the protein of interest. This assay technology was originally introduced to screen for type II and III kinase inhibitors in high-throughput formats[12] and since then has been successfully refined and transferred to other proteins.[13] We show that this fluorescent labels in nuclear receptors (FLiN) assay is a valuable tool to screen for agonists and antagonists of the human ERβ LBD in high-throughput formats and is suitable for distinguishing between the different conformations of helix 12 induced by the ligand.

To specifically label the ERβ LBD with a fluorophore, we introduced a cysteine residue in the flexible helix 12 by site-directed mutagenesis. From the crystal structures of the human ERβ LBD we identified four suitable sites (C478, D486C, L489C, N493C) for fluorophore labeling that lie at the beginning or within the C-terminal helix 12 (see Figure S1 for details on the construct design). Crystal structures suggest that a fluorophore attached to these positions will sense changes in the microenvironment upon binding of ligands that stabilize either a bent or straight conformation of helix 12, without interfering with ligand binding.

To ensure monolabeling of the FLiN protein constructs with the fluorophore, two solvent-exposed cysteine residues within the ERβ LBD were replaced by serine and leucine (C366S and C478L, respectively, according to a sequence alignment with related LBD sequences from a BLAST[14] search). Experiments with all four FLiN constructs, labeled with the environmentally sensitive fluorophore acrylodan, indicated that L489C was most sensitive to conformational changes and therefore the most promising mutant for FLiN (see Table S1 for Z' determinations). ESI-MS confirmed the correct labeling of the ERβ LBD (L489C) FLiN construct with acrylodan (Figure S2).

To verify that the substitutions introduced into the FLiN construct did not markedly alter the protein structure and stability, we recorded CD spectra of the FLiN construct (L489C) and wild-type ERβ LBD over a wide temperature range. Spectra for both proteins showed a high α-helical content and were comparable to previously reported CD spectra of the ER LBD (Figure S3).[15]

Furthermore, we analyzed the impact of the mutations in the FLiN construct (unlabeled) on ER activity using a standard fluorescence polarization assay (Figure S4) with the ER agonists E2 and diethylstilbestrol and the SERMs raloxifene, tamoxifen, and 4-hydroxytamoxifen as test compounds. Wild-type and the L489C mutant ERβ LBD showed comparable IC_{50} values in the nanomolar range for all tested compounds, indicating that the mutations had no vital effects on the binding affinities for known ligands. IC_{50} values obtained for the LBDs were slightly higher compared to the values for the wild-type full-length ERβ obtained with the same assay system. Data obtained for the full-length receptor were in the range of previously reported relative binding affinities for ERβ (18 ± 2 nM for E2, 15 ± 0 nM for diethylstilbestrol, and 73 ± 23 nM for raloxifene compared to 1–5.6 nM for E2,[16] 1.07–4 nM for diethylstilbestrol,[16b,c,17] and 12–557 nM for raloxifene[16c,18]).

We then examined the performance of the ERβ FLiN construct in the FLiN assay system in 384-well microtiter plates by measuring the emission spectra of acrylodan (λ_{ex}: 386 nm, λ_{em}: 410–560 nm) in the presence of increasing concentrations of the test compounds (Figure 2). In the unbound (apo) form, the spectrum showed a fluorescence maximum at 451 nm and a secondary maximum at 478 nm. Addition of the SERM 4-hydroxytamoxifen resulted in a bathochromic shift of the emission spectrum, with a significant decrease in the intensity at 451 nm and a shift of the second maximum from 478 nm to 500 nm. Notably, the addition of the agonist diethylstilbestrol resulted in a slight hypsochromic shift with an increase in the intensity at 478 nm. For E2, a similar but less pronounced trend could only be observed using a different buffer system without the addition of DTT (see the Supporting Information). A change of the buffer system did not notably affect the other test compounds.

These results indicated that the fluorescent label attached to helix 12 experiences diverse changes in its microenvironment upon the binding of different types of ligands as a bathochromic shift is linked to a more-solvent-exposed

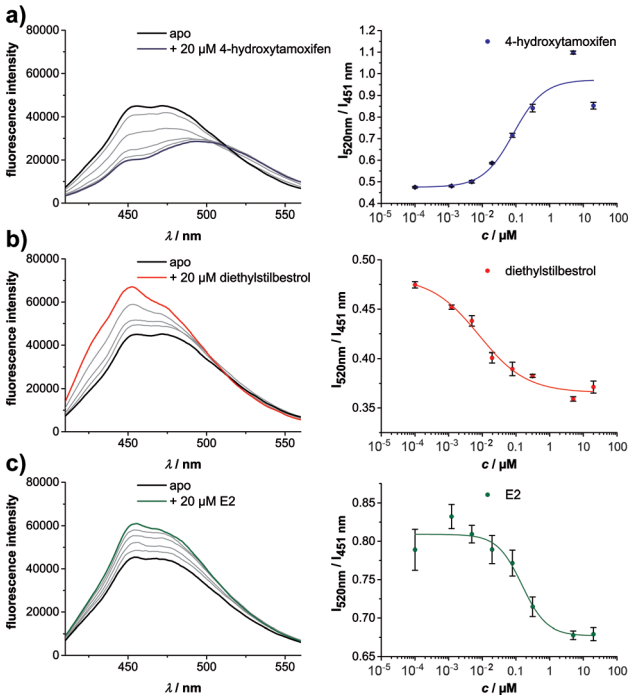

Figure 2. FLiN assay in 384-well format. Representative FLiN fluorescence spectra obtained with increasing concentrations of a) 4-hydroxytamoxifen, b) diethylstilbestrol, and c) E2 (spectra were smoothed; see Figure S5 for raw data). Increasing concentrations of ligand led to significant alterations in the emission spectrum. K_d values for the ligands were calculated by plotting the fluorescence intensity ratio $I_{520\,nm}/I_{451\,nm}$ against the logarithmic compound concentration.

environment.[19] Changes in the fluorescence spectrum can be used to calculate binding affinities for the tested ligands. This can be achieved by plotting the fluorescence intensity ratio ($r = I_{520\,nm}/I_{451\,nm}$) of the acrylodan emission spectrum against the logarithmic ligand concentration, leading to a sigmoidal binding curve where the inflection point corresponds to the K_d value. Different ligand binding modes translate into different output signals in the FLiN assay, depending on the conformation of helix 12 that is stabilized by the ligand in solution. For the SERM 4-hydroxytamoxifen, the intensity ratio increased with increasing compound concentrations, whereas the addition of diethylstilbestrol led to a decreasing signal.

We determined K_d values for the test compounds E2, diethylstilbestrol, raloxifene, tamoxifen, and 4-hydroxytamoxifen with the FLiN assay to be 88 ± 22 nM, 15 ± 5 nM, 1.3 ± 0.7 μM, 3.5 ± 2 μM, and 474 ± 310 nM, respectively ($n = 4$). Reported K_d values for ERβ were 0.4–2 nM for E2.[16b,20] No comparable K_d values from direct binding experiments are reported in the literature for ERβ with the other test compounds. K_d values from SPR experiments obtained with the ERα LBD are reported to be 0.009 nM for diethylstilbestrol, 220 nM for tamoxifen, and 18 nM for 4-hydroxytamoxifen.[21] Thus, K_d values determined with FLiN were higher, but show the same general trends as the results from competitive binding experiments. These differences could be due to the different protein constructs used in the assay systems and correlate with the higher K_d values obtained for the FLiN construct in our fluorescence polarization assay. However, the system is well suited as a screening assay for the detection and discrimination of compounds that stabilize active or inactive conformations of the receptor. The K_d values obtained with the FLiN assay were consistent over three individual protein preparations (individual expression, purification, and labeling of the receptor).

To assess the quality of the FLiN assay we determined the Z' value by measuring the fluorescence intensity ratio $I_{520\,nm}/I_{451\,nm}$ for a negative control (DMSO) and a positive control (20 μM diethylstilbestrol or 4-hydroxytamoxifen), each in eight replicates. Assay systems with a $Z' > 0.5$ are regarded to produce robust data in a high-throughput assay.[22] With a Z' of 0.75 ± 0.05 for diethylstilbestrol and 0.81 ± 0.09 ($n = 5$) for 4-hydroxytamoxifen, the FLiN assay is a valuable and robust tool for the detection of ERβ agonists and antagonists in high-throughput formats.

Furthermore, FLiN can also be used as a tool to monitor the real-time association and dissociation kinetics of ligands in cuvettes with a spectrofluorimeter (Figure 3). The binding

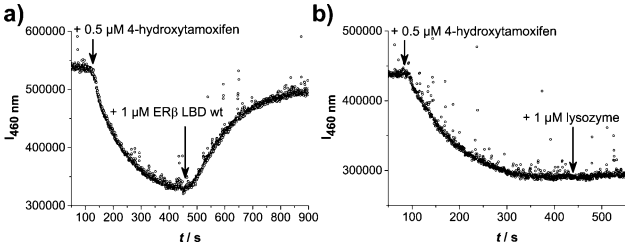

Figure 3. Association and dissociation of ligands were directly monitored by measuring time-resolved fluorescence intensity at 460 nm in cuvettes. a) Addition of 4-hydroxytamoxifen led to a slow association that was completely reversed by addition of excess unlabeled ERβ LBD wild-type. b) Addition of lysozyme as a negative control did not release the bound ligand (see Figure S7 for DMSO control).

of 4-hydroxytamoxifen was monitored by time-resolved measurements of the acrylodan fluorescence intensity at 460 nm, as the greatest intensity change was observed at this wavelength in the cuvette format (Figure S7). Addition of the test compound led to a slow decrease in the fluorescence intensity that corresponded to the association of compound and receptor. The ligand binding could be fully reversed by addition of excess unlabeled ERβ LDB wild-type, indicating that helix 12 underwent rearrangement after release of the ligand. As expected, the addition of an excess of lysozyme, as a negative control, did not release the bound ligand from the receptor. Further kinetic analyses were performed for 4-hydroxytamoxifen and diethylstilbestrol (Figure S8).

In summary, we report the development of a direct binding assay system for the identification of agonists and antagonists of the human ERβ in high-throughput formats. The FLiN assay produces robust data and distinguishes between different conformations of the LBD stabilized by the ligand. The FLiN assay is also a useful tool to directly monitor ligand binding kinetics without the need for additional instrumentation. We speculate that the presented FLiN assay system can be easily transferred to other nuclear

receptors and can also facilitate the identification of ligands for orphan nuclear receptors, which are considered to be interesting targets for current drug discovery efforts.[23]

Keywords: direct binding assay · drug discovery · estrogen receptor · fluorescence spectroscopy

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