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Monitoring ligand-mediated helix 12 transitions within the human estrogen receptor α using bipartite tetracysteine display†

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Estrogen receptor α ligand-binding domains (ERα-LBD) expressing tetracysteine motifs bind FlAsH-EDT$_2$ upon transition of helix 12 (H12) to a folded state. Changes in fluorescence intensity allowed surveillance of ligand-mediated H12 transitions and facilitated the determination of FlAsH association rates (k_{on}) and apparent equilibrium dissociation constants (K_{app}) to ERα-LBDs in the presence of estrogenic ligands.

The human estrogen receptor α (ERα) is a member of the nuclear receptor superfamily that functions as a ligand-mediated transcription factor. The ERα is activated by the natural steroid hormone 17β-estradiol (E2) and is responsible for regulating estrogen-mediated cellular processes such as growth, differentiation, transcription, signal transduction and apoptosis.[1,2] Dysregulated ERα activity has been linked to a number of diseases including cancer, diabetes, osteoporosis and neurodegeneration.[3] Consequently, the ERα is widely considered to be an important target for therapeutic intervention. The full-length ERα is comprised of five modular domains: an N-terminal transactivation domain, a DNA-binding domain, a hinge region, a ligand-binding domain (LBD) and a C-terminal domain.[4] Structurally, the ERα-LBD (30 kDa) is composed of 12 α-helices and a small β-strand arranged in a 'sandwich fold' that is conserved among members of the nuclear receptor superfamily.[5] The bulk of the ERα-LBD adopts a well-folded globular structure, however, the C-terminal helix 12 (H12, residues 538–548) is dynamic and can occupy multiple binding sites along the surface of the LBD. Crystallographic evidence has revealed that the structural configuration of H12 relative to the ERα-LBD is heavily influenced by the ligand occupying the binding pocket.[6] For example, when the receptor is bound to a pure ERα agonist such as E2, H12 packs against helices H3, H5/6 and H11 of the globular protein.[6] In this 'active conformation', H12 caps the ligand-binding site and facilitates binding of transcriptional co-activators to the ERα-LBD. Alternatively, when the ERα-LBD is bound to a selective estrogen receptor modulator (SERM) such as tamoxifen, H12 is unable to cap the ligand-binding site and repositions itself to bind the co-activator binding site between helices H3 and H5.[7] This so-called 'stable antagonist conformation' of H12 is thought to prevent dimerization and transcriptional activation of the receptor. Given the essential role H12 plays in regulating ERα activity, it is perhaps not surprising that there is considerable interest in evaluating how ligand-mediated H12 transitions affect ERα function. Indeed, researchers have utilized diverse strategies such as hydrogen deuterium exchange,[8] mass spectrometry,[9] spin labeling[10] and trypsin-coupled fluorescence polarization[11] to monitor H12 dynamics *in vitro*. These approaches typically require extensive chemical modification of the ERα-LBD, however, and are often difficult to apply in live cells. Cell-based transcriptional activation assays have also been employed to study ligand-mediated structural changes within the ERα-LBD,[12] although evaluating discrete H12 transitions was not the primary focus of these investigations. Beyond these methods, much of our current understanding about ligand-mediated H12 transitions within the ERα-LBD has been compiled from X-ray crystallography[6,13] and molecular modeling simulations.[14–16] While crystallographic studies have provided substantial information about the position of H12 in the presence of various estrogenic ligands, such structures present only a snapshot of the receptor co-complex and do not inform on the nature of H12 transitions in fluid environments. Furthermore, molecular dynamics simulations have been successful at modeling ligand-mediated H12 transitions under varied conditions, though the vast majority of these models have not yet been confirmed by experimental data.

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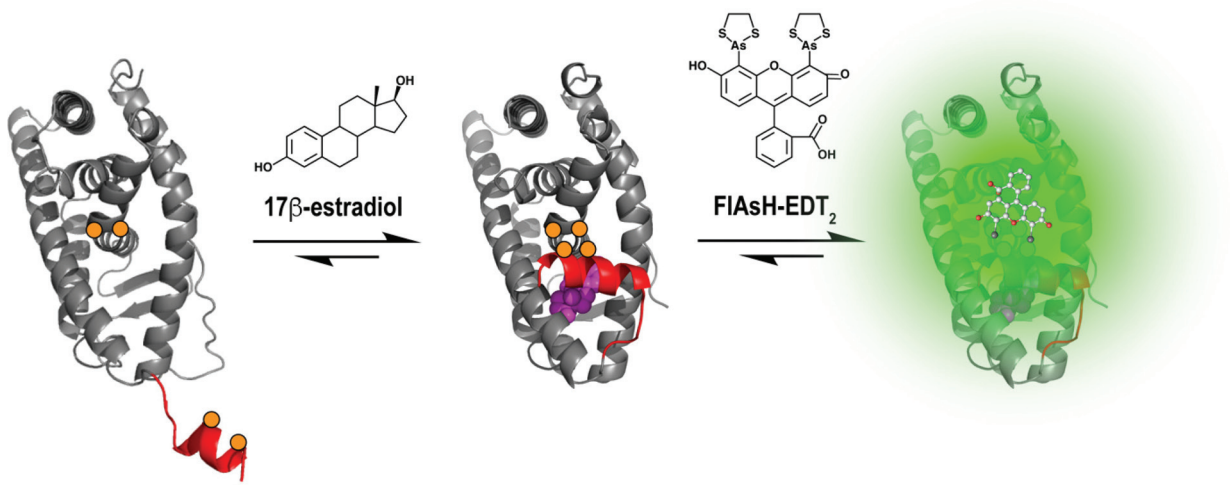

Fig. 1 Use of bipartite tetracysteine display to monitor ligand-mediated H12 transitions within the ERα-LBD. Unliganded receptor is shown with H12 extended, illustrating overall flexibility. Upon binding to 17β-estradiol (E2), H12 transitions to an active conformation that caps the ligand binding pocket. Subsequent binding of FlAsH to the C4 motif formed by the H12 transition results in fluorescence (green). H12 is shown in red; Cys residues are shown as orange circles; E2 is shown as pink spheres. Figures modeled from PDB IDs: 1A52 and 1ERE.

A practical approach for studying protein dynamics in real time is the use of genetically encodable tetracysteine (C4) tags coupled with biarsenical ligands such as FlAsH-EDT_2 or ReAsH-EDT_2.[17,18] This method relies on a specific, high-affinity interaction between a small molecule biarsenical pro-fluorophore and a protein sequence containing a C4 motif. Binding of FlAsH-EDT_2 to C4 motifs within proteins replaces the bound ethanedithiols (EDT) with cysteine thiols and converts an otherwise non-fluorescent molecule into a highly-fluorescent probe. It was reported in 2002 that such biarsenical dyes bind tightly to peptides that incorporate C4 motifs within the optimized peptide sequence CCPGCC.[19] Following this discovery, Schepartz and co-workers devised an elegant strategy known as 'bipartite tetracysteine display' that replaces the intervening PG sequence with a protein or protein interaction.[20] The notion here is that as long as the optimized C4 motif is approximated upon protein folding or association, FlAsH can be used to quantify such interactions as a function of fluorescence intensity. Importantly, the relatively small size of FlAsH in relation to the larger protein complex makes it ideal for studying structural dynamics without significantly impacting the kinetics of folding or association. Moreover, this method relies on incorporating natural (cysteine) amino acids to bind FlAsH and does not require any additional chemical modification of the protein or ligand. In this report, we apply bipartite tetracysteine display and FlAsH-EDT_2 to monitor ligand-meditated H12 transitions within the ERα-LBD *in vitro*.

Our efforts were initiated with the total chemical synthesis of FlAsH-EDT_2 [21] and confirmation of its pro-fluorescence using optimized control peptides (see ESI Fig. S1 and S2, Table S1†). We then focused our attention on the rational design of ERα-LBD mutants that could form an intramolecular C4 motif between H12 and the globular portion of the receptor upon ligand-mediated transition to an active conformation. We hypothesized that the protein–ligand interaction would trigger association of H12 with the LBD, converting an unfolded ERα-LBD with otherwise low affinity for FlAsH into a folded configuration that would bind FlAsH with high affinity (Fig. 1). To develop an optimized sequence for FlAsH binding, we surveyed the interfacial contacts between H12 and the LBD and identified discrete residues that could form a C4 motif upon conversion to an active conformation. After considering ERα-LBD crystal structures,[6,22] ligand-binding parameters[7,23,24] and mutagenesis studies,[25,26] four ERα-LBD variants were designed that allowed us to study ligand-mediated H12 transitions *in vitro*. All mutant receptors were generated using site-directed mutagenesis on plasmids coding for wild-type ERα-LBD (wtERα-LBD), with primers designed using Agilent web-based software (ESI Fig. S3, Tables S2 and S3†). The four mutants were engineered to express 0, 2 or 4 cysteines at different locations within their primary sequences (Fig. 2a). For these mutants, ERα-LBD-ΔC_0 contains no exposed cysteines to mitigate non-specific labeling;[27] ERα-LBD-ΔCC_N includes a dicysteine motif within H5 at positions 380 and 381; ERα-LBD-ΔCC_C expresses a dicysteine sequence within H12 at positions 546 and 547; and ERα-LBD-ΔC_4 includes four cysteines at positions 380, 381, 546 and 547. Importantly, the spacing of the C4 motif within the active conformation of ERα-LBD-ΔC_4 was determined to be similar to that of the optimized CCPGCC peptide sequence bound to ReAsH[28] (Fig. 2b).

All ERα-LBD mutants were expressed and purified from *E. coli* lysates (see ESI† for experimental details). To assess the impact of cysteine mutations on receptor structure, we evaluated our ERα-LBD proteins using wavelength-dependent circular dichroism (CD) spectropolarimetry (ESI Fig. S4†). Far-UV CD spectra at 20 °C confirmed that each ERα-LBD mutant adopts a well-folded, predominantly α-helical architecture that is reminiscent of the wtERα-LBD.[29] The thermal stability of

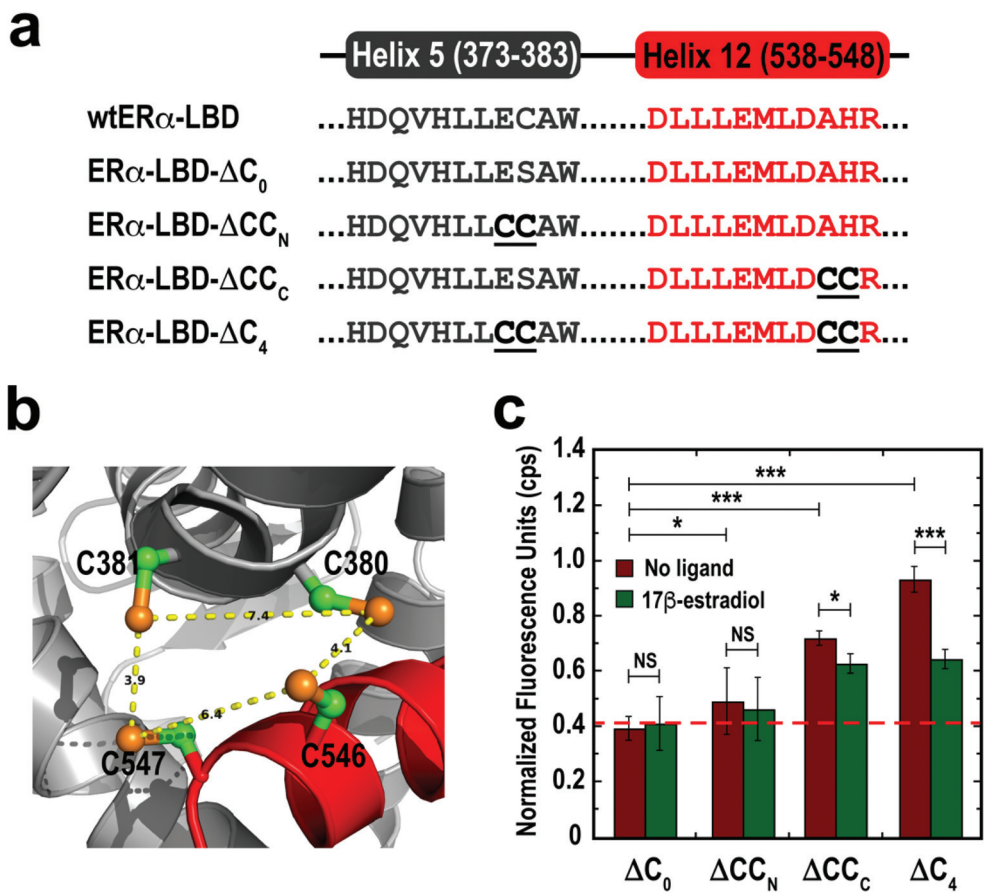

Fig. 2 (a) Sequences of ERα-LBD mutants used in this work; dicysteine motifs are shown underlined in bold. (b) Close-up of the optimized C4 motif formed between H12 and the ERα-LBD following ligand binding (active conformation). H12 is shown in red; the ERα-LBD is shown in grey; mutated Cys residues are shown as ball and stick; distances (Å) between Cys thiols are indicated by dotted yellow lines. (c) Endpoint fluorescence of solutions containing FlAsH-EDT_2 and ERα-LBD mutants. Values are displayed as normalized fluorescence units (counts per second, cps) and represent the average of three independent experiments. Dotted line indicates level of normalized FlAsH-EDT_2 fluorescence (0.419 cps) in the absence of protein. Error bars are standard deviation. * $p < 0.05$; *** $p < 0.005$; NS: not significant.

each mutant was then evaluated by monitoring the temperature-dependent CD signal at 222 nm. Here, we found that all mutants underwent cooperative melting transitions between 49.8 and 51.0 °C (ESI Table S4†). To determine whether our mutants could bind E2, we employed a fluorescence polarization competitive binding assay using a fluorescent E2-derivative (ESI Fig. S5 and Table S4†). These experiments showed that ERα-LBD-ΔC_0 and ERα-LBD-ΔC_4 bound E2 with respective IC_{50} values of 7.6 nM and 2.8 nM, which are similar to E2 binding affinities observed for the wtERα-LBD *in vitro*.[30] These results suggested that the absence of exposed cysteines or the presence of a C4 motif does not significantly affect the ability for the ERα-LBD to bind natural ligand. ERα-LBD-ΔCC_N and ERα-LBD-ΔCC_C were shown to bind E2 with respective IC_{50} values of 68.1 nM and 456.3 nM, representing a 24- and 163-fold decrease in affinity compared to ERα-LBD-ΔC_4. While these observations indicate that isolated dicysteine motifs along H5 and H12 moderately reduce the affinity of ERα-LBDs for E2, each mutant bound the natural ligand with IC_{50} values that were suitable for succeeding assays.

Next, we evaluated whether FlAsH could target our mutant ERα-LBDs and produce a measurable change in fluorescence upon complexation with cysteine thiols in the absence of ligand. For these experiments, we incubated our ERα-LBD variants with FlAsH-EDT_2 in binding buffer (see ESI† for experimental details) and subsequently measured the endpoint fluorescence of the solution (Fig. 2c). All FlAsH-based association experiments described herein were performed in binding buffer supplemented with 10 μM EDT to minimize non-specific labeling of exposed dithiols by FlAsH-EDT_2.[20,31,32] It was observed here that ERα-LBD-ΔC_0 exhibited fluorescence counts that were similar to samples containing FlAsH-EDT_2 alone. This result indicates that FlAsH does not target ERα-LBDs that are devoid of solvent-exposed cysteines and that the receptor itself does not contribute to the background fluorescence intensity of the sample. In contrast, dicysteine-containing mutants ERα-LBD-ΔCC_N and ERα-LBD-ΔCC_C each exhibited non-negligible increases in fluorescence when compared to ERα-LBD-ΔC_0. Specifically, ERα-LBD-ΔCC_N showed a 1.3-fold increase in fluorescence compared to ERα-LBD-ΔC_0 in

the absence of ligand, while ERα-LBD-ΔCC_C exhibited a nearly 2-fold increase compared to ERα-LBD-ΔC_0 under similar conditions. Taken together, these data suggest that FlAsH can bind to dicysteine motifs that are positioned within the globular portion of the LBD (H5), but binds more effectively to dicysteine thiols that are located within H12. These results are supported by the previous finding that FlAsH can target exposed dithiols in isolation, albeit with markedly less fluorescence than when bound to a C4 motif.[32,33] Of all mutants tested, the ERα-LBD-ΔC_4 variant showed the brightest fluorescence in the absence of ligand. This construct exhibited a fluorescence intensity that was nearly 3-fold higher than that observed with ERα-LBD-ΔC_0 and 1.3-fold higher than ERα-LBD-ΔCC_C. This result suggests that ERα-LBDs containing C4 motifs emit higher fluorescence counts when bound to FlAsH compared to proteins containing isolated dithiols. At this point, we noted that biarsenical profluorophore reagents such as FlAsH-EDT_2 can crosslink individual peptides and proteins containing dicysteine sequences.[19,20,32] We therefore hypothesized that the fluorescence observed in unliganded samples may arise not only from FlAsH binding to the organized C4 motif, but also from FlAsH-mediated crosslinking through dithiols located within H5 or H12. In order to distinguish additional modes of association between FlAsH and ERα-LBD-ΔC_4, we performed in-gel fluorescence analysis[19] of labeled receptor complexes and monitored the gels for higher molecular weight species. As expected, in-gel fluorescence was not observed for ERα-LBD-ΔC_4 proteins treated without FlAsH-EDT_2 (data not shown). Conversely, ERα-LBD-ΔC_4 proteins treated with FlAsH-EDT_2 showed obvious fluorescent bands around 30 kDa, indicating that FlAsH can efficiently label the monomeric species of this receptor in the absence of ligand (ESI Fig. S6†). In addition to the labeled monomeric bands, we also observed a sharp fluorescent band near 60 kDa in lanes containing unliganded ERα-LBD-ΔC_4. We reasoned that this upper band was a dimeric form of the receptor that was crosslinked by FlAsH through dicysteine motifs located within the ERα-LBD-ΔC_4 protein. Comparative analysis of the monomeric and dimeric fluorescent band intensities revealed that this sample contained 12.5% crosslinked receptor, indicating that a non-negligible amount of fluorescence emanates from this specific mode of FlAsH binding. Finally, to determine whether FlAsH-mediated crosslinking of ERα-LBD-ΔC_4 is promoted through dicysteine motifs located within H5 or H12, we treated ERα-LBD-ΔCC_N and ERα-LBD-ΔCC_C with FlAsH-EDT_2 and attempted to visualize dimeric forms of the receptors using in-gel fluorescence. Under these conditions, we observed no fluorescent bands at 60 kDa in samples containing unliganded ERα-LBD-ΔCC_N and a strong fluorescent band at 60 kDa in samples containing ERα-LBD-ΔCC_C (data not shown). This result indicates that FlAsH-mediated crosslinking of ERα-LBD-ΔC_4 is facilitated through dicysteine motifs located within H12 and not those within the globular portion of the receptor (H5).

Following this preliminary evaluation of how FlAsH associates with unliganded receptors, we shifted our attention to assessing how FlAsH targets our ERα-LBD mutants complexed with E2. For these experiments, we pre-treated each ERα-LBD variant with E2 and incubated the protein–ligand complexes with FlAsH-EDT_2 (Fig. 2c). Here, it was observed that the E2-bound forms of ERα-LBD-ΔC_0 and ERα-LBD-ΔCC_N exhibited the same fluorescence as their unliganded counterparts. This result suggests that E2 does not influence the ability for FlAsH to complex with these specific ERα-LBD mutants. On the other hand, we noticed that E2-bound ERα-LBD-ΔCC_C showed a significant decrease in fluorescence compared to its unliganded construct. This observation indicates that binding of E2 to ERα-LBD-ΔCC_C causes a structural change in the receptor that effectively mitigates binding of FlAsH to the isolated H12 dithiol. Surprisingly, we found that E2-bound ERα-LBD-ΔC_4 receptors exhibited nearly 30% lower fluorescence compared to their unliganded forms (Fig. 2c). Initially, we anticipated that FlAsH would bind the E2-bound receptor with higher affinity and display enhanced fluorescence over unliganded ERα-LBD-ΔC_4. Instead, FlAsH complexed to E2-bound ERα-LBD-ΔC_4 showed less overall fluorescence, suggesting that E2 elicits a change in H12 conformation that ultimately reduces the intensity of the FlAsH tracer. To rule out possible quenching from the ligand, we performed FlAsH binding experiments with control peptides (see ESI† for peptide sequences) in the presence or absence of 10 μM E2. Here, we determined that the fluorescence intensities of both samples were essentially identical, indicating that solvated E2 does not quench fluorescence or mitigate FlAsH complexation with the control peptide (data not shown). While we cannot entirely rule out the possibility that quenching of the fluorophore occurs when both FlAsH and E2 are bound to ERα-LBD-ΔC_4, the fact that the phenolic (A) ring of the complexed E2 would be positioned over 18 Å away from the bound FlAsH molecule makes quenching under these conditions highly unlikely.[6,34] We therefore speculated that the reduced fluorescence seen with the E2-bound receptors was a result of H12 being tightly associated with the LBD and precluding FlAsH-EDT_2 from undergoing efficient thiol-arsenic exchange with the C4 motif. To gain further insight into this conjecture, we used in-gel fluorescence to determine whether E2-bound receptors are subject to FlAsH-mediated crosslinking through extended H12 regions (ESI Fig. S6†). Comparative analysis of fluorescent band intensities at 30 kDa revealed that unliganded ERα-LBD-ΔC_4 was 41% brighter than that of its E2-bound counterpart, suggesting that FlAsH labels ERα-LBD-ΔC_4 monomers more efficiently in the absence of ligand. We also observed that the 60 kDa band in the E2-treated lanes was noticeably fainter compared to corresponding bands in samples containing no ligand, indicating that the extent of crosslinking was markedly reduced in the presence of E2. Indeed, quantification of the fluorescent band intensities showed that only 2.6% of the E2-bound receptors were crosslinked under these conditions. These results suggest that binding of E2 causes a significant conformational change in the ERα-LBD that prevents FlAsH-mediated crosslinking through H12 and is consistent with the model that E2 forces H12 into an active conformation that is tightly associated with the receptor. More subtly, however,

these findings suggested to us FlAsH-mediated crosslinking may be used to qualitatively assess whether H12 can adopt extended conformations when bound to various ERα ligands.

Encouraged by these findings, we applied FlAsH-based kinetic, titration and crosslinking experiments to study how various estrogenic analogs affect ligand-mediated H12 transitions *in vitro*. For these studies, we determined the association rate (k_{on}), apparent equilibrium dissociation constant (K_{app}) and crosslinking ability (%) of FlAsH for ERα-LBD-ΔC_4 proteins complexed with E2, ethynyl estradiol (EE2) and fulvestrant (FUL). EE2 is a synthetic estrogen that acts as an ERα agonist and is primarily used in hormone replacement therapies and contraceptives.[35] FUL is a pure ERα antagonist that exerts its physiological effects by preventing ERα dimerization and accelerating degradation of the receptor *in vivo*.[36] In addition, FUL is thought to inhibit H12 from binding in an active conformation to the ERα-LBD.[37] To evaluate the kinetics of FlAsH association, we incubated FlAsH-EDT$_2$ with ERα-LBD-ΔC_4 in the presence or absence of each ligand and measured fluorescence increase as a function of time (Fig. 3a). All estrogenic ligands used in these studies were incubated with ERα-LBD-ΔC_4 at 10 μM to ensure complete binding to the receptor. This concentration was chosen as it is well-above the reported IC_{50} values for E2 (0.89 nM), EE2 (0.47 nM) and FUL (2.4 nM) when targeting the ERα-LBD *in vitro*.[38] In all cases, formation of the FlAsH complex with ERα-LBD-ΔC_4 resulted in a measurable fluorescence increase that reached a plateau after two-hours of incubation. Kinetic data were fitted to a single exponential (see ESI† for details), which was used to calculate k_{on} values of FlAsH to the liganded and unliganded receptors (Table 1). The k_{on} for FlAsH to unliganded ERα-LBD-

Table 1 Association rates (k_{on}), apparent equilibrium dissociation constants (K_{app}) and dimerization percentages (%) for FlAsH and ERα-LBD-ΔC_4 proteins in the presence or absence of estrogenic ligands

Ligand	k_{on} [a] (M^{-1} min^{-1})	K_{app} [b] (μM)	Dimer[c] (%)
No ligand	36 236	0.68 ± 0.58	29.8
17β-Estradiol	44 590	1.64 ± 1.03	8.4
Ethynyl estradiol	38 090	1.05 ± 1.01	9.3
Fulvestrant	38 637	2.47 ± 1.92	29.4
4-Hydroxytamoxifen	39 182	3.02 ± 1.82	28.8
Raloxifene	57 036	2.91 ± 2.00	40.0
Bazedoxifene	47 294	1.76 ± 0.55	42.4

[a] Values calculated from data in Fig. 3, panels a and c. [b] Values calculated from data in Fig. 3, panels b and d. [c] Values calculated from in-gel fluorescence Fig. S7.†

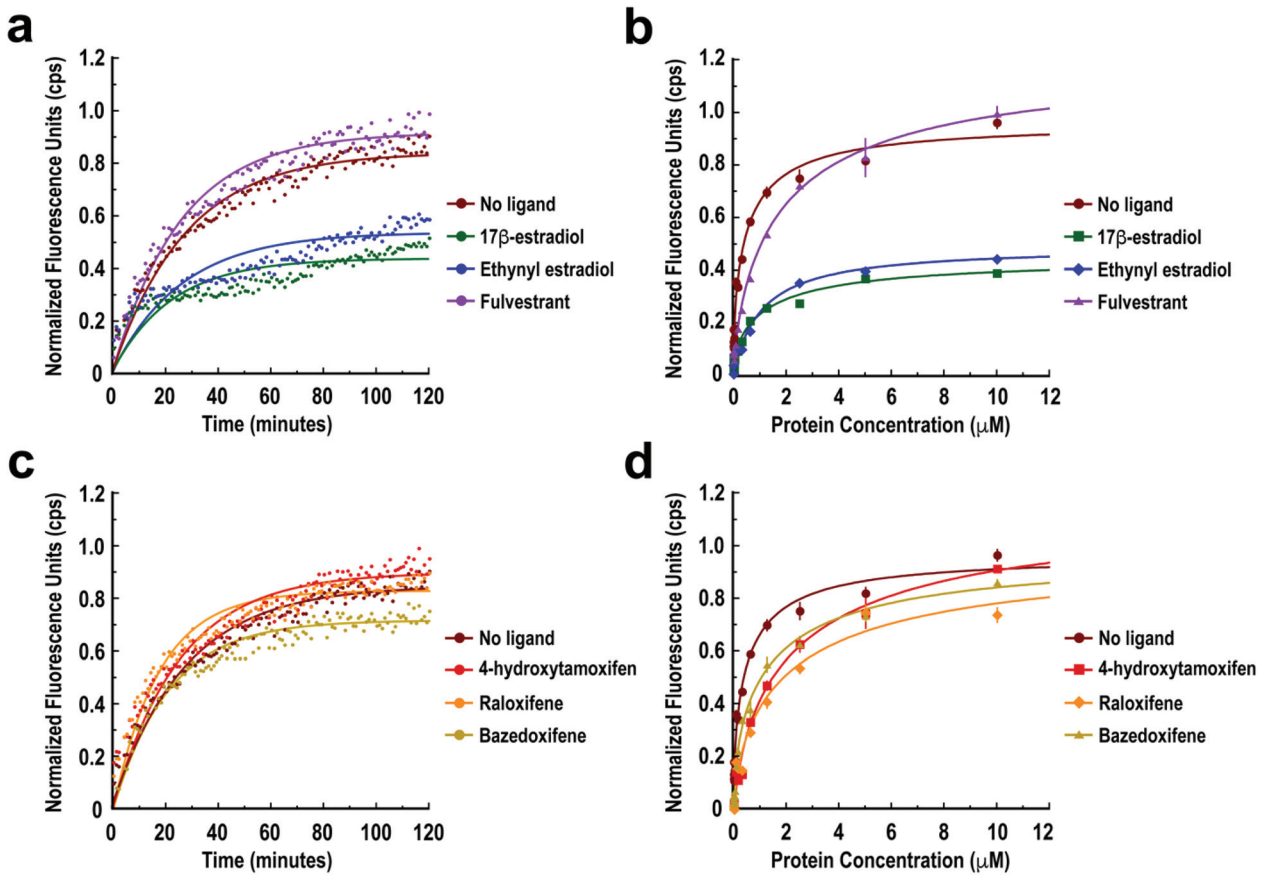

Fig. 3 (a) Initial rates of association and (b) equilibrium binding between FlAsH and ERα-LBD-ΔC_4 in the presence or absence of estrogenic ligands. (c) Initial rates of association and (d) equilibrium binding between FlAsH and ERα-LBD-ΔC_4 in the presence or absence of SERM ligands. For kinetic experiments, data points were collected every minute for 120 minutes and are displayed as normalized fluorescence units (cps); all plots show the normalized fluorescence units (cps) of FlAsH solutions upon the addition of liganded or unliganded ERα-LBDs. Error bars are standard deviation.

ΔC_4 was determined to be 36 236 M^{-1} min^{-1}, while the k_{on} for the E2-bound form was 44 590 M^{-1} min^{-1}. In contrast, incubating the receptor with EE2 or FUL resulted in respective k_{on} values of 38 090 M^{-1} min^{-1} and 38 637 M^{-1} min^{-1}. These results suggest that FlAsH binds to the C4 motif of the E2-bound isoform at a faster rate compared to unliganded receptors or receptors bound to EE2 or FUL. Despite a moderately faster association rate, however, E2-bound receptors displayed lower overall fluorescence intensities compared to unliganded, EE2- and FUL-bound forms at all timepoints tested. This is consistent with the lower fluorescence intensities observed for E2-bound receptors in our endpoint and crosslinking experiments (Fig. 2c, ESI Fig. S6†). We next determined the apparent equilibrium dissociation constant (K_{app}) of FlAsH for liganded and unliganded ERα-LBD-ΔC_4 using fluorescence titration experiments. For these assays, we incubated FlAsH-EDT_2 with ERα-LBD-ΔC_4 in the presence or absence of ligand and quantified sample fluorescence as a function of protein concentration (Fig. 3b). Here, it was observed that unliganded ERα-LBD-ΔC_4 bound FlAsH with a K_{app} of 0.68 ± 0.58 μM, while FlAsH targeted the E2-, EE2- and FUL-complexed forms with respective K_{app} values of 1.64 ± 1.03 μM, 1.05 ± 1.01 μM and 2.47 ± 1.92 μM (Table 1). These results suggest that under these conditions, FlAsH has the highest affinity for unliganded ERα-LBD-ΔC_4 and the lowest affinity for receptors bound to FUL. Furthermore, these data show that FlAsH has slightly higher affinity for receptors bound to EE2 compared to those complexed with natural ligand. Finally, we quantified FlAsH-mediated crosslinking of ERα-LBD-ΔC_4 proteins bound to each estrogenic ligand by performing in-gel fluorescence experiments (Table 1, ESI Fig. S7†). As expected, unliganded ERα-LBD-ΔC_4 showed a moderate amount of crosslinking (29.8%), while crosslinking among E2-bound receptors was substantially diminished (8.4%). It was also observed that EE2-bound receptors displayed a similar level of crosslinking (9.3%) to those bound to E2 and that FUL-bound forms were crosslinked at the same percentage (29.4%) as unliganded receptors (Table 1).

The combined results from these experiments allowed us to reach several important conclusions about how the presence or absence of estrogenic ligands affect H12 dynamics within the ERα-LBD *in vitro.* For example, FlAsH displayed the highest affinity and slowest association rate for unliganded ERα-LBD-ΔC_4. We interpret these results as H12 being highly dynamic in the absence of ligand, forming a somewhat loose active conformation C4 motif that can be targeted by FlAsH with high affinity once organized in the proper configuration. Moreover, nearly 30% of the unliganded receptors were found to be crosslinked under these conditions, indicating that H12 can adopt an extended structure that facilitates FlAsH-mediated crosslinking in the absence of ligand. Similar association rates and receptor crosslinking percentages were observed with ERα-LBD-ΔC_4 proteins bound to FUL, suggesting that the extent of H12 flexibility is similar in unliganded and FUL-bound receptors. Nevertheless, FUL-bound ERα-LBD-ΔC_4 exhibited a 4-fold decrease in K_{app} compared to unliganded proteins, which may be attributed to the inability for H12 to form a fully-folded active conformation C4 motif in the presence of the pure antagonist.[39] Notably, FlAsH was found to associate most rapidly with ERα-LBD-ΔC_4 receptors that were bound to E2. This observation, coupled with the modestly low K_{app} of FlAsH for the E2-bound receptor, indicates that FlAsH can bind favorably to the C4 motif that is formed upon transition of H12 to an active conformation. FlAsH was also observed to have a slightly lower K_{app} for ERα-LBD-ΔC_4 proteins bound to EE2, but associated with this complex at a slower rate compared to receptors bound to natural ligand. Collectively, these results suggest that EE2 causes H12 to adopt a structural conformation that is similar to the E2-bound form, however, the ethynyl group of EE2 may cause some slight structural perturbations within the receptor that slow the rate at which H12 transitions to an active conformation. Finally, we observed that FlAsH-mediated crosslinking was significantly reduced in receptors treated with E2 or EE2. This finding suggested to us that these ligands force H12 into a tight association with the LBD that effectively mitigates crosslinking through extended H12 domains. Furthermore, the inability to crosslink though H12 likely contributes to the reduced overall fluorescence observed in these samples compared to those containing unliganded or FUL-bound complexes.

We next evaluated ligand-mediated H12 transitions in the context of various SERM ligands, including 4-hydroxytamoxifen (4HT), raloxifene (RAL) and bazedoxifene (BAZ). 4HT is the active metabolite of tamoxifen, a widely-used anticancer drug that acts as a mixed ERα agonist/antagonist.[40] RAL and BAZ are mixed ERα agonists/antagonists that are primarily used to treat osteoporosis in post-menopausal women.[40] Crystallographic evidence has revealed that each of these small-molecules bind the ERα-LBD within the ligand-binding site that is naturally occupied by E2.[6,7] However, the extended structural architectures of these SERMs cause them to protrude slightly from the binding pocket and block H12 from forming a well-organized active conformation with the receptor. In the presence of such ligands, H12 is reoriented to bind a hydrophobic groove (the co-activator binding site) located between helices H3 and H5 on the ERα-LBD.[6,7] This stable antagonist conformation elicited by SERM compounds is thought to affect modified or reduced ERα activity in certain cell types.[6,40]

We applied similar *in vitro* FlAsH-based assays as described above to evaluate how 4HT, RAL and BAZ affect H12 transitions within the ERα-LBD. To determine the kinetics of FlAsH binding in the context of these SERM ligands, we incubated FlAsH-EDT_2 with ERα-LBD-ΔC_4 complexed to 4HT, RAL or BAZ and measured fluorescence increase as a function of time (Fig. 3c). As with our estrogenic ligands, all SERMs were incubated with ERα-LBD-ΔC_4 at 10 μM to ensure complete binding to the receptor. This concentration was again chosen as it is well-above the respective IC_{50} values for 4HT (5.13 nM),[38] RAL (2.4 nM)[41] and BAZ (26 nM)[41] when targeting the ERα-LBD *in vitro*. It was observed here that all treatments

resulted in a measurable increase in fluorescence that reached a plateau within two hours. Additionally, it was noted that each complex resulted in a maximal fluorescence that was similar to samples containing unliganded receptor. All kinetic data were fitted to a single exponential (see ESI† for details) that was used to calculate k_{on} values of FlAsH to the SERM-bound receptors (Table 1). Specifically, the association rate for FlAsH to 4HT-bound ERα-LBD-ΔC_4 was found to be 39 182 M^{-1} min^{-1}, which is similar to k_{on} values observed with unliganded and FUL-bound complexes. The respective association rates for FlAsH to RAL- and BAZ-bound receptors were 57 036 M^{-1} min^{-1} and 47 294 M^{-1} min^{-1}, indicating that FlAsH is able to target these specific complexes more rapidly than unliganded or 4HT-bound forms. We next used fluorescence titration experiments to evaluate K_{app} of FlAsH for ERα-LBD-ΔC_4 in the presence and absence of SERM ligands. For these studies, we pre-incubated ERα-LBD-ΔC_4 with 4HT, RAL or BAZ and subsequently treated each sample with FlAsH-EDT_2. We then plotted the level of FlAsH fluorescence as a function of protein concentration (Fig. 3d). These studies revealed that FlAsH had the highest affinity for BAZ-bound ERα-LBD-ΔC_4 proteins with a K_{app} of 1.76 ± 0.55 μM. It was also observed that FlAsH targeted 4HT- and RAL-bound receptors with similar affinities, having K_{app} values of 3.02 ± 1.82 μM and 2.91 ± 2.00 μM respectively (Table 1). Notably, we found that all SERM ligands resulted in a relatively high level of FlAsH-mediated crosslinking of ERα-LBD-ΔC_4. Specifically, 4HT showed 28.8% crosslinking, while RAL and BAZ each showed respective crosslinking percentages of 40.0% and 42.4% under these conditions.

The results from these experiments showed that the association rate, apparent equilibrium dissociation constant and dimerization efficiency of FlAsH is heavily influenced by which SERM is complexed to the ERα-LBD. Ultimately, we found that each SERM caused H12 to adopt a discrete structural configuration that has a unique interaction profile with FlAsH. For example, FlAsH had the slowest association rate and lowest affinity to receptors bound to 4HT, indicating that this ligand forces H12 into a conformation that is not optimized to bind FlAsH. On the other hand, RAL seemed to cause H12 to adopt a structure that allowed rapid association of FlAsH to the receptor, albeit with a relatively low binding affinity. Receptors bound to BAZ showed moderate association rates with FlAsH and had the lowest K_{app} value out of all SERMs evaluated under these conditions. Notably, each SERM ligand resulted in modest to high levels of receptor crosslinking, with RAL and BAZ showing the greatest percentage of dimers out of all ligands tested. These results imply that H12 remains flexible and can adopt extended structures even in the presence of SERM-based ligands that drive the ERα-LBD into stable antagonist conformations.

Conclusions

In summary, we have utilized bipartite tetracysteine display coupled with FlAsH-EDT_2 to develop a novel system for monitoring ligand-mediated H12 transitions within the ERα-LBD *in vitro*. We present this approach as a proof-of-principle to observe how discrete interactions between the ERα-LBD and select estrogenic ligands affect the position and flexibility of H12 as it relates to the globular portion of the receptor. The value of this technique is apparent in that it provides an entirely genetically encodable, fluorescence-based assay that can be used to monitor ligand-mediated H12 transitions within the ERα-LBD in real time. What is more, this strategy does not require any chemical modification of the ERα-LBD or the ligand itself, allowing for minimal impact on the natural ligand–receptor interaction. The studies outlined herein demonstrated that this technique has high temporal resolution, which enabled us to determine kinetics of association for FlAsH to ERα-LBDs complexed with different ligands. We also applied fluorescence titration assays to quantify the K_{app} values of FlAsH for each ERα-LBD in the context of various estrogenic compounds. Such experiments were used to infer an equilibrium value for FlAsH binding and all fluorescence data were interpreted strictly as empirical measurements of H12 conformational dynamics. As an additional advantage, in-gel fluorescence allowed us to observe FlAsH-mediated crosslinking of ERα-LBDs, which was construed as a qualitative indication of H12 flexibility.

It should be noted that FlAsH-mediated crosslinking does not necessarily bear any relation to physiological dimerization of the ERα-LBD, particularly since crosslinking is observed when the receptor is bound to compounds that elicit stable antagonist conformations. Nevertheless, there is evidence indicating that antagonists such as FUL and 4HT provide greater kinetic stabilization of dimeric receptors over ERα agonists *in vitro*.[27] It is therefore possible that higher incidences of FlAsH-mediated crosslinking are observed with FUL, 4HT, RAL and BAZ because a greater percentage of receptors are physiologically dimerized when bound to these ligands. While this possible correlation is interesting and certainly warrants future study, it should not affect the interpretation of our results, as H12 flexibility can be determined by our system regardless of the dimeric state of the receptor.

Taken together, our results indicate that H12 remains flexible when the ERα-LBD is bound to FUL, 4HT, RAL or BAZ. These findings suggest that the presumed stable antagonist conformation elicited by SERM-based compounds is, in fact, dynamic and that H12 can adopt extended conformations even when bound to such ligands. Contrarily, ERα-LBD agonists such as E2 and EE2 appear to drastically reduce the proportion of extended H12 by forcing the helix to associate with the receptor in a 'tight' active conformation. Going forward, we seek to employ our FlAsH-based system to study how clinically relevant mutations within the ERα-LBD affect H12 dynamics in the presence or absence of ligand. For instance, Y537S and D358G mutations within the ERα-LBD enable hormone-free transcriptional activity and result in reduced agonist potency.[42] Work in our laboratory is currently focused on repeating our FlAsH-based assays in the context of these mutations. Finally, the successful application of bipartite tetra-

cysteine display to monitor ligand-mediated H12 transitions within the ERα-LBD suggests that this approach may be useful for studying helix transitions in other members of the nuclear receptor superfamily and may be applied to visualize ligand-mediated H12 dynamics in living cells.

Conflicts of interest

The authors declare no competing financial interest.

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References

1 L. Bjornstrom and M. Sjoberg, Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes, *Mol. Endocrinol.*, 2005, **19**, 833–842.

2 C. Brisken and B. O'Malley, Hormone action in the mammary gland, *Cold Spring Harbor Perspect. Biol.*, 2010, **2**, a003178.

3 B. J. Deroo and K. S. Korach, Estrogen receptors and human disease, *J. Clin. Invest.*, 2006, **116**, 561–570.

4 P. Yasar, G. Ayaz, S. D. User, G. Gupur and M. Muyan, Molecular mechanism of estrogen-estrogen receptor signaling, *Reprod. Med. Biol.*, 2017, **16**, 4–20.

5 Y. Li, M. H. Lambert and H. E. Xu, Activation of nuclear receptors: a perspective from structural genomics, *Structure*, 2003, **11**, 741–746.

6 A. M. Brzozowski, A. C. Pike, Z. Dauter, R. E. Hubbard, T. Bonn, O. Engstrom, L. Ohman, G. L. Greene, J. A. Gustafsson and M. Carlquist, Molecular basis of agonism and antagonism in the oestrogen receptor, *Nature*, 1997, **389**, 753–758.

7 A. K. Shiau, D. Barstad, P. M. Loria, L. Cheng, P. J. Kushner, D. A. Agard and G. L. Greene, The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen, *Cell*, 1998, **95**, 927–937.

8 S. Y. Dai, T. P. Burris, J. A. Dodge, C. Montrose-Rafizadeh, Y. Wang, B. D. Pascal, M. J. Chalmers and P. R. Griffin, Unique ligand binding patterns between estrogen receptor alpha and beta revealed by hydrogen-deuterium exchange, *Biochemistry*, 2009, **48**, 9668–9676.

9 D. A. Seielstad, K. E. Carlson, P. J. Kushner, G. L. Greene and J. A. Katzenellenbogen, Analysis of the structural core of the human estrogen receptor ligand binding domain by selective proteolysis/mass spectrometric analysis, *Biochemistry*, 1995, **34**, 12605–12615.

10 K. M. Hurth, M. J. Nilges, K. E. Carlson, A. Tamrazi, R. L. Belford and J. A. Katzenellenbogen, Ligand-induced changes in estrogen receptor conformation as measured by site-directed spin labeling, *Biochemistry*, 2004, **43**, 1891–1907.

11 A. Tamrazi, K. E. Carlson and J. A. Katzenellenbogen, Molecular sensors of estrogen receptor conformations and dynamics, *Mol. Endocrinol.*, 2003, **17**, 2593–2602.

12 X. Gu, Helix 12 in the human estrogen receptor (hER) is essential for the hER function by overcoming nucleosome repression in yeast, *J. Cell. Biochem.*, 2002, **86**, 224–238.

13 R. Kumar, M. N. Zakharov, S. H. Khan, R. Miki, H. Jang, G. Toraldo, R. Singh, S. Bhasin and R. Jasuja, The dynamic structure of the estrogen receptor, *J. Amino Acids*, 2011, **2011**, 812540.

14 L. Celik, J. D. Lund and B. Schiott, Conformational dynamics of the estrogen receptor alpha: molecular dynamics simulations of the influence of binding site structure on protein dynamics, *Biochemistry*, 2007, **46**, 1743–1758.

15 F. Fratev, Activation helix orientation of the estrogen receptor is mediated by receptor dimerization: evidence from molecular dynamics simulations, *Phys. Chem. Chem. Phys.*, 2015, **17**, 13403–13420.

16 M. T. Sonoda, L. Martinez, P. Webb, M. S. Skaf and I. Polikarpov, Ligand dissociation from estrogen receptor is mediated by receptor dimerization: evidence from molecular dynamics simulations, *Mol. Endocrinol.*, 2008, **22**, 1565–1578.

17 H. Gelman, A. J. Wirth and M. Gruebele, ReAsH as a Quantitative Probe of In-Cell Protein Dynamics, *Biochemistry*, 2016, **55**, 1968–1976.

18 G. E. Sosinsky, G. M. Gaietta, G. Hand, T. J. Deerinck, A. Han, M. Mackey, S. R. Adams, J. Bouwer, R. Y. Tsien and M. H. Ellisman, Tetracysteine genetic tags complexed with biarsenical ligands as a tool for investigating gap junction structure and dynamics, *Cell Commun. Adhes.*, 2003, **10**, 181–186.

19 S. R. Adams, R. E. Campbell, L. A. Gross, B. R. Martin, G. K. Walkup, Y. Yao, J. Llopis and R. Y. Tsien, New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications, *J. Am. Chem. Soc.*, 2002, **124**, 6063–6076.

20 N. W. Luedtke, R. J. Dexter, D. B. Fried and A. Schepartz, Surveying polypeptide and protein domain conformation and association with FlAsH and ReAsH, *Nat. Chem. Biol.*, 2007, **3**, 779–784.

21 S. R. Adams and R. Y. Tsien, Preparation of the membrane-permeant biarsenicals FlAsH-EDT2 and ReAsH-EDT2 for

fluorescent labeling of tetracysteine-tagged proteins, *Nat. Protoc.*, 2008, **3**, 1527–1534.
22 F. Rastinejad, P. Huang, V. Chandra and S. Khorasanizadeh, Understanding nuclear receptor form and function using structural biology, *J. Mol. Endocrinol.*, 2013, **51**, T1–t21.
23 S. W. Fanning, L. Hodges-Gallagher, D. C. Myles, R. Sun, C. E. Fowler, I. N. Plant, B. D. Green, C. L. Harmon, G. L. Greene and P. J. Kushner, Specific stereochemistry of OP-1074 disrupts estrogen receptor alpha helix 12 and confers pure antiestrogenic activity, *Nat. Commun.*, 2018, **9**, 2368.
24 D. M. Tanenbaum, Y. Wang, S. P. Williams and P. B. Sigler, Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 5998–6003.
25 S. Aliau, H. Mattras, E. Richard and J. L. Borgna, Cysteine 530 of the human estrogen receptor alpha is the main covalent attachment site of 11beta-(aziridinylalkoxyphenyl) estradiols, *Biochemistry*, 1999, **38**, 14752–14762.
26 M. Gangloff, M. Ruff, S. Eiler, S. Duclaud, J. M. Wurtz and D. Moras, Crystal structure of a mutant hERalpha ligand-binding domain reveals key structural features for the mechanism of partial agonism, *J. Biol. Chem.*, 2001, **276**, 15059–15065.
27 A. Tamrazi, K. E. Carlson, J. R. Daniels, K. M. Hurth and J. A. Katzenellenbogen, Estrogen receptor dimerization: ligand binding regulates dimer affinity and dimer dissociation rate, *Mol. Endocrinol.*, 2002, **16**, 2706–2719.
28 F. Madani, J. Lind, P. Damberg, S. R. Adams, R. Y. Tsien and A. O. Graslund, Hairpin structure of a biarsenical-tetracysteine motif determined by NMR spectroscopy, *J. Am. Chem. Soc.*, 2009, **131**, 4613–4615.
29 N. Greenfield, V. Vijayanathan, T. J. Thomas, M. A. Gallo and T. Thomas, Increase in the stability and helical content of estrogen receptor alpha in the presence of the estrogen response element: analysis by circular dichroism spectroscopy, *Biochemistry*, 2001, **40**, 6646–6652.
30 K. Ohno, T. Fukushima, T. Santa, N. Waizumi, H. Tokuyama, M. Maeda and K. Imai, Estrogen receptor binding assay method for endocrine disruptors using fluorescence polarization, *Anal. Chem.*, 2002, **74**, 4391–4396.
31 B. A. Griffin, S. R. Adams and R. Y. Tsien, Specific covalent labeling of recombinant protein molecules inside live cells, *Science*, 1998, **281**, 269–272.
32 B. Krishnan and L. M. Gierasch, Cross-strand split tetra-Cys motifs as structure sensors in a beta-sheet protein, *Chem. Biol.*, 2008, **15**, 1104–1115.
33 K. Stroffekova, C. Proenza and K. G. Beam, The protein-labeling reagent FLASH-EDT2 binds not only to CCXXCC motifs but also non-specifically to endogenous cysteine-rich proteins, *Pflugers Arch.*, 2001, **442**, 859–866.
34 N. Gajadeera and R. N. Hanson, Review of fluorescent steroidal ligands for the estrogen receptor 1995-2018, *Steroids*, 2019, **144**, 30–46.
35 H. Kuhl, Pharmacology of estrogens and progestogens: influence of different routes of administration, *Climacteric*, 2005, **8**(Suppl 1), 3–63.
36 R. W. Carlson, The history and mechanism of action of fulvestrant, *Clin. Breast Cancer*, 2005, **6**(Suppl 1), S5–S8.
37 X. Long and K. P. Nephew, Fulvestrant (ICI 182,780)-dependent interacting proteins mediate immobilization and degradation of estrogen receptor-alpha, *J. Biol. Chem.*, 2006, **281**, 9607–9615.
38 R. M. Blair, H. Fang, W. S. Branham, B. S. Hass, S. L. Dial, C. L. Moland, W. Tong, L. Shi, R. Perkins and D. M. Sheehan, The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands, *Toxicol. Sci.*, 2000, **54**, 138–153.
39 A. C. Pike, A. M. Brzozowski, J. Walton, R. E. Hubbard, A. G. Thorsell, Y. L. Li, J. A. Gustafsson and M. Carlquist, Structural insights into the mode of action of a pure antiestrogen, *Structure*, 2001, **9**, 145–153.
40 S. Martinkovich, D. Shah, S. L. Planey and J. A. Arnott, Selective estrogen receptor modulators: tissue specificity and clinical utility, *Clin. Interventions Aging*, 2014, **9**, 1437–1452.
41 B. S. Komm, Y. P. Kharode, P. V. Bodine, H. A. Harris, C. P. Miller and C. R. Lyttle, Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity, *Endocrinology*, 2005, **146**, 3999–4008.
42 S. W. Fanning, C. G. Mayne, V. Dharmarajan, K. E. Carlson, T. A. Martin, S. J. Novick, W. Toy, B. Green, S. Panchamukhi, B. S. Katzenellenbogen, E. Tajkhorshid, P. R. Griffin, Y. Shen, S. Chandarlapaty, J. A. Katzenellenbogen and G. L. Greene, Estrogen receptor alpha somatic mutations Y537S and D538G confer breast cancer endocrine resistance by stabilizing the activating function-2 binding conformation, *eLife*, 2016, **5**, e12792.