Implications of the binding of tamoxifen to the coactivator recognition site of the estrogen receptor

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Abstract

A number of studies have reported on the unusual pharmacological behavior of type I antiestrogens, such as tamoxifen. These agents display mixed agonist/antagonist activity in a dose-, cell-, and tissue-specific manner. Consequently, many efforts have been made to develop so-called ‘pure’ antiestrogens that lack mixed agonist/antagonist activity. The recent report of the structure of estrogen receptor (ER) β with a second molecule of 4-hydroxytamoxifen (HT) bound in the coactivator-binding surface of the ligand-binding domain (LBD) represents the first direct example of a second ER ligand-binding site and provides insight into the possible origin of mixed agonist/antagonist activity of type I antiestrogens. In this review, we summarize the biological reports leading up to the structural conformation of a second ER ligand-binding site, compare the ERβ LBD structure bound with two HT molecules to other ER structures, and discuss the potential for small molecular inhibitors designed to directly inhibit ER-coactivator and, more generally, nuclear receptor (NR)-coactivator interactions. The studies support a departure from the traditional paradigm of drug targeting to the ligand-binding site, to that of a rational approach targeting a functionally important surface, namely the NR coactivator-binding (activation function-2) surface. Furthermore, we provide evidence supporting a reevaluation of the strict interpretation of the agonist/antagonist state with respect to the position of helix 12 in the NR LBD.

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Background

Estrogen biology is exceedingly complex and important in the development and function of numerous tissues and physiological phenomena. Estrogen receptor (ER) α and β proteins display ubiquitous yet differential expression in many tissues, including adipose, adrenal, bone, brain, breast, colon, inner ear, liver, lung, pancreas, pituitary, sympathetic ganglia, thyroid, and urogenital (Diel 2002, Nilsson & Gustafsson 2002, Gustafsson 2003, Matthews & Gustafsson 2003, Heldring et al. 2007b). Accordingly, estrogen signaling affects the cardiovascular, central nervous, immune, musculoskeletal, and reproductive systems, and manifests in a plethora of physiological symptoms and diseases, perhaps most notable of which is breast cancer. ERα and ERβ have both overlapping and unique functions (Matthews & Gustafsson 2003), and ERβ is thought to act as an inhibitor of ERα activity (Hall & McDonnell 1999). Knowledge of the tissue-specific expression and the subsequent role of ERα and ERβ proteins in disease will provide opportunities to develop novel treatments for colon cancer, depression, hearing disorders, infertility, leukemia, osteoporosis, prostate and endometrial cancers, and others (Gustafsson 2003, Matthews & Gustafsson 2003).

ERα and ERβ are members of the nuclear receptor (NR) superfamily and have evolutionarily conserved modular structures (Mangelsdorf et al. 1995, Renaud & Moras 2000). The N-terminal activation function-1 (AF-1) region is responsible for ligand-independent transcriptional activation of NR proteins and contains multiple phosphorylation sites. The DNA-binding domain is C-terminal to the AF-1 region and functions
Biochemical history of the dual agonist/antagonist activity of type I antiestrogens

A number of studies have reported that type I antiestrogens display both agonist and antagonist properties in a dose-, cell-, tissue-, and species-dependent manner. In MCF-7 (Katzenellenbogen et al. 1987, Berthois et al. 1994) and ZR-75-1 (Poulin et al. 1989) human breast cancer cells, as well as rat uterus (Black et al. 1983), the administration of low concentrations of the type I antiestrogen tamoxifen results in a stimulatory (agonist) effect on cell growth. Higher tamoxifen concentrations, however, result in a conversion from agonist to antagonist activity (inhibition of cell growth). Depending on the particular species and tissue studied, tamoxifen exhibits properties of a pure agonist, a partial agonist/antagonist, or a pure antagonist (Jordan 1984). For example, tamoxifen displays mixed agonist/antagonist behavior in human and rat tissues, but under certain circumstances is a pure agonist in mouse and guinea pig (Furr & Jordan 1984, Jordan 1984). Tamoxifen also displays differential effects in human tissues, where it is an antagonist in breast and an agonist in uterine and bone tissues (Gottardis et al. 1988, Love et al. 1992, McDonnell 1999). By contrast, type II antiestrogens, including U11110A, LY 117018, and ICI 164 384, display exclusively antagonist behavior.

Type I and type II antiestrogens were shown to expose an ER epitope specific for the monoclonal activity. Furthermore, ER was shown to bind nearly twice as much antiestrogen when compared with E₂ (Hedden et al. 1995). These studies and others led to the proposal of a unified two-site model describing the action of antiestrogens (Jensen & Khan 2004). This model relates the agonist activity of type I antiestrogens to the occupancy of the cognate ligand-binding site, whereas antagonist activity results from an additional interaction with a secondary location. Recently, the proposal of a second ligand-binding site was corroborated with the report of a crystal structure of ERβ LBD bound to two molecules of 4-hydroxytamoxifen (HT; Wang et al. 2006). Herein, we briefly describe the history of the pharmacological behavior of type I antiestrogens and support for a second ER ligand-binding site, building up to the structural model describing the second binding site for HT within the LBD of ERβ. Furthermore, we discuss the implications of the second binding site in the inhibition of ER/NR-coactivator interactions, as well as in the definition of the agonist or antagonist state based on the position of helix 12 in the LBD.
antibody H222 (Martin et al. 1988, Berthois et al. 1994, Hedden et al. 1995). These studies, which were carried out in the presence of a saturating amount of E2, led to the hypothesis that exposure of the H222-specific epitope results from the interaction of the antiestrogens at a distinct site from the cognate ligand-binding site. This alleged second ligand-binding site appeared to have a lower affinity for ligand, when compared with the cognate ligand-binding site for E2. In support, sucrose sedimentation and tritiated ligand (Fig. 1A) experiments revealed that ER from MCF-7 cytosol binds approximately twice the amount of type I (HT) or type II (RU 58 668) antiestrogens when compared with E2 when studied in the low nM range (Hedden et al. 1995). Interestingly, the concentration at which the second site-binding event occurs correlates to approximately the same concentration where agonism changes to antagonism (Fig. 1B; Katzenellenbogen et al. 1987).

Another study raised the possibility of a second ligand-binding site for HT, as a high degree of ‘nonspecific’ HT binding was observed (Raam et al. 1983). This study reported an HT:ER binding ratio of 1.7 ligand per receptor using a dextran-coated charcoal assay – the same assay and approximate binding ratio reported in an aforementioned study (Hedden et al. 1995). The authors state that it was not possible to determine whether HT is binding to the E2-binding site or another functionally relevant site, but that it was in fact the same protein moiety as the E2-binding site. By contrast, the type II antiestrogen RU 58 668, which has a lower affinity for ER when compared with E2 (Van de Velde et al. 1994), does not display a concentration-dependent change from agonism to antagonism, affording a sole antagonist role. This was suggested to occur via two approximately simultaneous binding events for RU 58 668 (Jensen & Khan 2004). However, binding of type II compounds to the cognate ligand-binding site is likely sufficient to induce an antagonist response. Structural studies (discussed in a later section) reveal that the steroid portion of these compounds binds in the cognate ligand-binding pocket, whereas the bulky R group component of these compounds associates in the AF-2 surface (also discussed later as the possible second ligand-binding site). In this scenario, low concentrations of ligand resulting in binding to the cognate-binding site would be sufficient to induce an antagonist response. Higher ligand concentrations may therefore result in binding to an additional second site, but because the primary binding event causes antagonism, no change in pharmacological behavior is observed. Of note, it has also been reported that E2 can expose an ER epitope specific for the H222 monoclonal antibody at seemingly high concentrations that resulted in a decrease in the stimulatory activity of E2 (Berthois et al. 1994).

Analysis of ER from the cytosol of ovariectomized guinea pigs (Hedden et al. 1995) revealed a substance...
that sediments more slowly than the ER-ligand complex and associates with HT but not E₂. The authors suggest that the concentration of free (tamoxifen) ligand is reduced such that secondary binding is not achievable, hence the sole agonist action. Similar observations have been made, for example, in cytosol extracts from chick oviduct (Sutherland & Foo 1979, Sutherland et al. 1980), mature rat uterus (Faye et al. 1980, Sutherland et al. 1980), immature rat uterus (Sutherland et al. 1980, Murphy & Sutherland 1981a,b), rat liver (Watts & Sutherland 1984, 1986, van den Koedijk et al. 1992), rat serum (Winneker et al. 1983), guinea pig uterus (Gulino & Pasqualini 1980), calf uterus (Lerea et al. 1987), ER-positive human breast carcinomas (Sutherland et al. 1980, Jordan et al. 1981, Murphy & Sutherland 1981a,b, Watts et al. 1984, Berthois et al. 1994), human follicular thyroid carcinoma cells (Gross et al. 1993), and human endometrium (Sutherland et al. 1980). In these tissues, a biomacromolecule was observed to bind tamoxifen and other estrogenic derivatives, including ICI 47699 (Murphy & Sutherland 1981a), and displays tissue-specific differences in cellular concentration (Winneker & Clark 1983). This substance was subsequently named the microsomal antiestrogen-binding site (AEBS); a hetero-oligomeric multiprotein complex found in most mammals in different tissue-dependent concentrations (Mésange et al. 2002, Kedjouar et al. 2004). For example, AEBS is ~20–30 times more abundant in the liver when compared with tumor cell lines (Chailleux et al. 1994). It has been suggested that AEBS regulates the free intracellular concentrations of antiestrogens (Kudolo et al. 1984). More recently, it was reported that non-antiestrogen ligands targeting AEBS increase the anti-proliferative activity of antiestrogens (End et al. 2001, Baum & Kirschmeier 2003, de Bono et al. 2003, Dalenc et al. 2005). It has been further suggested that prolonged exposure to antiestrogens may result in the production of AEBS (Furr & Jordan 1984), implying that the inhibition of AEBS antiestrogen binding resulting in increased cellular bioavailability of antiestrogens. Tamoxifen-sensitive, but not tamoxifen-insensitive, cell lines show immunoreactivity to the H222 antibody (Naundorf et al. 2000) and, interestingly, no differences in the ER nucleotide sequences of the ER LBD in tamoxifen-sensitive and -insensitive cell lines were observed. This suggests that either sequences removed from the LBD or other biomacromolecules, such as AEBS or perhaps CtIP (Wu et al. 2007), may impart tamoxifen sensitivity directly or indirectly. This notion is further supported by the observation that ER mutations occur at a low frequency in breast cancer patients and do not account for most tamoxifen-resistant breast tumors (Karnik et al. 1994, Naundorf et al. 2000). When taken together with the possibility that AEBS displays differential and tissue-specific concentrations and its possible role in regulating intracellular antiestrogen concentrations, it is tempting to speculate that perhaps AEBS is present at different concentrations in tamoxifen-sensitive (lower) and tamoxifen-insensitive (higher) cell lines, resulting in the observed dose-, cell-, and tissue-specific mixed agonist/antagonist activity. Therefore, it may be the case that small molecular inhibitors of AEBS that prevent it from binding tamoxifen may allow tamoxifen-insensitive cell lines to respond to tamoxifen treatment. Further work is needed to determine the effects of estrogens and antiestrogens on H222 reactivity and delineate a possible role for AEBS in regulating the intracellular concentrations of these compounds with respect to ER function.

Altogether, these studies support a unified two-site model for antiestrogen activity (reviewed in Jensen & Khan 2004), which associates the agonist activity of type I antiestrogens to occupancy of the E₂-binding site, and the antagonist activity to an additional interaction with a secondary location. This model suggests that the mixed agonist/antagonist activity is a consequence of the weak ligand affinity for the second (antagonist) ligand-binding site for type I antagonists, when compared with the cognate (agonist) ligand-binding site. We note other studies reporting results in corroboration of the two-site antiestrogen, or more generally, antihormone model for ER/NR action. Low concentrations of E₂ and bisphenol A induced a stimulatory/proliferative response in rat cerebellum (Zsarnovszky et al. 2005). However, increasing concentrations were less efficacious and resulted in the inhibition of cellular proliferation. The authors suggested a model in which ‘the rapid-acting ER complex contains a high-affinity (picomolar) stimulatory binding site and a lower affinity (nanomolar) inhibitory binding site’ (Zsarnovszky et al. 2005). In another study, a significant dose-dependent increase in H222 immunoreactivity was observed in tamoxifen-sensitive MaCa 3366 ER/progesterone receptor (PR)-positive mammary carcinoma cells (Naundorf et al. 2000). Finally, it was reported that a mutant of the androgen receptor (AR) was unable to bind the agonist ligand progesterone but able to bind the antagonist ligand RU 486, suggesting a distinct binding site on AR for RU 486 but not progesterone (Vegeto et al. 1992, Gao & McPhaul 1998).

To the best of our knowledge, no additional, conclusive biochemical studies of ER antiestrogen
stoichiometry have been reported in support of, or opposition to, the two-site model. However, we note a number of studies reported with either unique or somewhat conflicting observations. For example, a set of studies revealed that one molecule of HT, but not E₂, out of two total molecules, dissociates from the ER dimer after binding to the estrogen response element (ERE; Klinge et al. 1996a,b, 1998), perhaps via interaction with an accessory protein that interacts with E₂-bound, but not HT-bound, ER (Anolik et al. 1996). In the aforementioned study in support of the two-site antiestrogen model (Raam et al. 1983), other assays performed report a HT:ER ratio of ~1. However, the authors suggest that this value may be due to more extensive dissociation of ligand during washes. The data suggest that not all but some population of ER proteins had two bound HT ligands. It is tempting to speculate that this may be a result of a weaker affinity, second binding site. In other studies, the dependence of HT or E₂ on the binding of ER to nuclei extracted from cellular tissues was analyzed without mention of a specific ligand:protein stoichiometry measurement (Cushing et al. 1985, Klinge et al. 1987, 1989). Additionally, visual inspection of data that report a 1:1 ratio for HT-bound ER via Scatchard analysis (Klinge et al. 1989) suggests a more complex binding curve. For cases like these where Scatchard analysis is used, which often results in errors when determining the number of binding sites (Klotz 1982, Faguet 1986), computer software utilizing a nonlinear regression analysis would be more appropriate for fitting equations and extracting rate parameters. Furthermore, in the aforementioned studies, as well as those not mentioned here, it is sometimes difficult to note whether the use of ‘nonspecific binding’, which implies low-affinity binding sites, is the same as ‘background signal’, as the two are not the same. In any case, additional studies aimed at probing the binding stoichiometry of agonists and type I/II antiestrogens and the resulting pharmacological phenotype are warranted. However, crystallographic structural studies described below provide additional support for a second, antagonistic ligand-binding site for the type II compound tamoxifen.

Structural validation of a second NR ligand-binding site

The first structural confirmation of a second NR ligand-binding site was revealed in the report of the structure of human ERβ LBD bound to two molecules of HT (Wang et al. 2006). The overall fold of HT-ERβ LBD (Fig. 2) is similar to previously published ER/NR LBD structures, namely a three-layered α-helical sandwich. One HT molecule is located in the cognate ligand-binding pocket, whereas the second HT molecule is located in the hydrophobic groove of the coactivator-binding (AF-2) surface, comprising helices 3, 4, 5, and 12. This surface provides a binding site for coactivator proteins containing the LXXLL recognition motif (Savkur & Burris 2004). The binding of the second HT molecule in the AF-2 surface primarily involves hydrophobic and van der Waals interactions. The unsubstituted phenyl group of the second HT molecule is buried deep into the hydrophobic cavity of the coactivator surface, which was noted to likely provide the main contribution to binding in the AF-2 surface. Consistent with the aforementioned biological data suggesting a second low-affinity binding site, the second HT molecule has higher crystallographic temperature factors and is not fully buried when compared with the HT molecule in the cognate ligand-binding site. The charge clamp residues Lys314 and Glu493, which are important for recognition of the LXXLL motif, are not involved in the interaction with the second HT molecule. We note that ERβ residues involved in binding the second HT molecule are highly conserved in ERα and ERβ proteins in other species (Fig. 3). Again we note that although, for example, tamoxifen is a pure agonist in mouse under certain circumstances and tissues (Furr & Jordan 1984, Jordan 1984), the tamoxifen-binding complex AEBS displays differential tissue-specific concentration that may not allow the intracellular concentration of tamoxifen to saturate the lower affinity second binding site in some tissues if present at sufficient concentrations.

The structural conformation of the LBD helix 12 is thought to directly influence the agonist/antagonist active state and NR coactivator binding. A number of structural studies have provided insight into the possible mechanism of agonist or antagonist ligand-induced activity of ER proteins (reviewed in Pike 2006). Binding of ER agonist ligands (e.g. E₂ and DES) results in a conformation whereby helix 12 caps the ligand-binding site, leaving the AF-2 surface exposed for coactivator binding (Fig. 4A and B). ER antagonist ligands induce a conformation resulting in unfavorable conditions for coactivator binding. Some antagonists have bulky components that protrude from the ligand-binding site that directly influence coactivator binding through direct contacts with the AF-2 surface (Fig. 4C), whereas others function in a passive manner through a lack of appropriate contacts in the ligand-binding cavity. The binding of passive antagonists is thought to alter the positioning of
helix 12, rotating it ~90° from the agonist position, allowing it to occupy the AF-2 coactivator binding surface (Fig. 4D). A partial agonist is thought to induce an intermediate conformation (Fig. 4E), whereby a suitable coactivator interaction can dynamically switch helix 12 between partial and full agonist positions.

The position of helix 12 in the ERβ LBD structure with a second HT molecule bound in the AF-2 surface (Fig. 4F) is atypical compared with other ER structures bound to agonist, partial agonist, and antagonist molecules (Wang et al. 2006). Additionally, there is a secondary interaction between the second HT molecule and helix 12 from a neighboring ERβ LBD molecule in the crystal lattice. The authors note that the alternative positions of helix 12 are not uncommon among other NR LBD structures (Tanenbaum et al. 1998, Gangloff et al. 2001, Pike et al. 2001, Xu et al. 2002, Wu et al. 2005). In particular, the secondary helix 12 interaction was noted to have a similar conformation to that of helix 12 in the structure of peroxisome proliferator-activated receptor α (PPARA) LBD bound to the antagonist ligand GW6471 and the NCOR peptide (Fig. 4G; Xu et al. 2002). The significance of the position of helix 12 in the HT-ERβ LBD structure remains elusive. However, the degree to which it is displaced and the secondary helix 12 interaction with the second HT molecule leads to the speculation that its novel placement could provide a means of coregulator binding through the specific recognition of the second HT molecule in the AF-2 surface.

We note that the crystal structure of ERα LBD bound to HT does not show a second HT molecule bound to the AF-2 surface (Shiau et al. 1998). The ERβ LBD structure bound to two HT molecules (Wang et al. 2006) was determined from protein that had ten times the molar concentration of the protein in the crystallization buffer. By contrast, ligand-bound ERα LBD (Shiau et al. 1998) was obtained by eluting the protein from an E2-affinity column with the ligand of interest (Greene et al. 1980) – a commonly used method to purify ERα LBD protein for crystallography (Goldstein et al. 2001). The authors do not specify if ligand was included during the crystallization process; therefore, it is possible that the second binding site in this study was unoccupied due to the unavailability of additional ligand at saturating conditions to bind to the AF-2 surface. This is supported by the notion that the binding of HT to the AF-2 surface is of lower affinity compared with the cognate ligand-binding pocket (Wang et al. 2006).

Recently, it was reported that the Y537S ERα LBD mutation allows for the conformational stabilization during protein purification and subsequently allows for the addition of ligands to purified apoprotein (Nettles et al. 2008). More recently, structural studies of AR (Estébanez-Perpiñá et al. 2007a) and thyroid hormone receptor β (THRβ; Estébanez-Perpiñá et al. 2007b) LBDs were reported, which corroborate the concept of a non-cognate ligand-binding site in LBD of NR proteins. The structure of AR LBD was determined bound to natural ligand (dihydrotestosterone; DHT) and small molecules found to inhibit AR/coregulator interactions – [4-(4-hydroxy-3-iodo-phenoxy)-3,5-diiodo-phenyl]-acetic acid, 4HY; 3,5,3’triiodothyronine, T3. The small molecules bind in the AF-2 surface (referred to as BF-2) and a nearby surface (BF-3), which comprises helix 1, the helix 3–5 loop, and helix 9 (Fig. 4H and I). The BF-3 surface is nearly the same size as the AF-2 region and is a known target for mutations in prostate cancer and androgen insensitivity syndrome. The small molecules bind to the AR LBD with IC50 ~50 μM and weaken the binding of coactivator peptides, suggesting that small molecules binding to sites other than the cognate ligand-binding site can modulate NR-coactivator interactions. In the
case of THRB (Estébanez-Perpiñá et al. 2007b), a high-throughput screen identified the aromatic β-enone 1-(4-hexylphenyl)-prop-2-en-1-one (HPPE) molecule as an inhibitor of steroid receptor coactivator-2 (SRC-2) interaction, and crystallographic studies revealed that HPPE binds to the AF-2 surface of THRB. Indeed, the authors confirm that molecules targeted to the AF-2 regions have a great potential in modulating NR coregulator recruitment.

Additional studies describing an antagonist-specific NR ligand-binding site

Other biochemical and molecular modeling studies have investigated the existence of a second NR ligand-binding site. The fluorescent ligand 5,6,11,12-tetrahydrocrysene ketone (THCK) was found to bind E2-ligated and -unliganded ERα and ERβ, suggesting a second E2-independent binding site (Tyulmenkov & Klinge 2000). Fluorescence experiments monitoring the binding of
THCK, which is an agonist for ERα and an antagonist for ERβ, suggested the molecule induced different ERα and ERβ conformations, providing a possible origin for the modes of agonist and antagonist action. A molecular modeling study was conducted to determine the location of THCK binding (van Hoorn 2002). Structures of the LBD of ERα and ERβ were analyzed for surface cavities and grooves, and three sites of interest were identified (Fig. 5): 1) the conserved, cognate ligand-binding site, 2) the AF-2 coactivator-binding site, and 3) a site proximal to the steroid-binding site comprising the helix 2/β-sheet region. The results of this study, however, were inconclusive for providing a basis by which THCK binding to the helix 2/β-sheet region could result in agonist properties for ERα and antagonist properties for ERβ.

In another study, the observation that the 1α,25(OH)₂-vitamin D₃ (1,25D) analogs do not compete well with [³H]1,25D, but did induce agonist/antagonist non-genomic properties, suggested the existence of an alternative vitamin D receptor (VDR) ligand-binding site (Mizwicki et al. 2004). Molecular modeling studies identified a possible alternative ligand-binding site, termed the A-pocket (alternative ligand-binding pocket). The A-pocket comprises a surface analogous to the aforementioned ER helix 2/β-sheet region (van Hoorn 2002) and was postulated to accept ligands of different shape compared with the G-pocket (genomic pocket), which is analogous to cognate ER ligand-binding site (Rochel et al. 2000, Tocchini-Valentini et al. 2001, Norman et al. 2004). The authors suggest the A-pocket affords a rapid, non-genomic response, distinct from the genomic response afforded by the G-pocket.

Although these studies indeed support the concept of an additional ligand-binding site for ER and VDR proteins, the proposed site (helix 2/β-sheet; Fig. 5) differs from the second ligand-binding site in the AF-2 surface reported for AR (Este´banez-Perpin´a et al. 2007a), ERβ (Wang et al. 2006), and THRB (Este´banez-Perpin´a et al. 2007b). It is possible that this site represents an additional ligand-binding site, but this remains to be experimentally verified. Recently, the helix 2/β-sheet surface in ERα was shown to be a previously unidentified ligand-independent coregulator interaction site (Kong et al. 2005). The authors propose that this site is highly unlikely to bind natural small molecule ligands. In support of this study, targeted mutations to this site in ERα, which is evolutionarily conserved among other members of the NR superfamily, resulted in reduced ligand-binding affinity (cognate site) and reduced SRC-1 interaction (Raviscioni et al. 2006).

**Figure 4** Positioning of helix 12 and ligand-binding site locations in various NR protein structures. (A) E2SR1 (agonist; PDB: 1ERE) (Brzozowski et al. 1997), (B) DES–ERα–GRIP complex (agonist; PDB: 3ERD) (Shiau et al. 1998), (C) ICI 164 384–ERβ (antagonist; PDB: 1HJ1) (Pike et al. 2001), (D) HT–ERα (passive antagonist; PDB: 3ERT) (Shiau et al. 1998), (E) genistein–ERβ (partial agonist; PDB: 1QKM) (Pike et al. 1999), (F) HT–ERβ (second HT-binding site; PDB: 2FSZ) (Wang et al. 2006), (G) GW6471–PPARA–NCOR complex (PDB: 1KKQ) (Xu et al. 2002), (H) DHT/4HY–AR (PDB: 2PIU) (Este´banez-Perpin´a et al. 2007a), (I) DHT/T₃–AR (PDB: 2PIW) (Este´banez-Perpin´a et al. 2007a), and (J) E₂–ERα–E₂#23 FN3 monobody (PDB: 2OCF). Ligands (space-filled molecules) bound in the cognate ligand-binding site are colored red, whereas ligands bound in other regions are green (AF-2/BF-2 or BF-3) or yellow (other locations). Helix 12 is colored blue, whereas bound peptides and helix 12 from a neighboring molecule HT-ERβ and the E₂#23 FN3 monobody are colored orange. In (G) and (H), the AR C-terminal extension of H12, which forms β-sheet contacts to the helix 9–helix 10 loop, is highlighted with a dotted oval.
Furthermore, studies of PPARG (Bruning et al. 2007), retinoid X receptor α (RXRA; Yan et al. 2004, 2006, Nahoum et al. 2007), and ERα (Dai et al. 2008) reveal that the binding of partial agonists in the cognate-ligand binding site results in a change in dynamics in the helix 2/β-sheet region. Changes in conformational dynamics have been shown to correlate positively to regions important for allosteric control of protein function (generally discussed in Kern & Zuiderweg 2003, Busenlehner & Armstrong 2005, Swain & Gierasch 2006, Henzler-Wildman & Kern 2007), further supporting the concept that the helix 2/β-sheet region is a control surface in NR proteins. Notwithstanding these results, the possibility exists for the development of small molecules to modulate the function of this surface.

Inconsistencies between the two-site antagonist model and ER LBD structures highlight the importance of the F domain

The generally accepted model for ligand-induced agonism or antagonism, as described above, relates the position of helix 12 to the active state, namely the ability to bind coactivator protein. Based on this model, structural studies of the ER LBD domain prior to the HT-ERβ structure (Wang et al. 2006) suggest that one molecule of the antiestrogen HT bound in the cognate ligand-binding pocket is sufficient to induce an antagonist AF-2 conformation and, thus, antagonist ER action (Shiau et al. 1998). This, however, is inconsistent with the biological data supporting the two-site model for antiestrogen action (Jensen & Khan 2004). We again note that different crystallization conditions were used in studies of the single HT-bound ERα and ERβ LBD bound to two HT molecules (Shiau et al. 1998, Wang et al. 2006). The two-site model associates the agonist activity of type I antiestrogens, such as HT, to occupancy of the E2-binding site, and the antagonist activity results from an additional interaction with a secondary location. Again, this is supported by studies that reveal that ER can bind nearly twice as much antiestrogen compared with E2, and that the second binding event of the type I antiestrogen HT correlates to a switch from agonism to antagonism (Fig. 1).

The discrepancy between the two-site antiestrogen model and the agonist/antagonist position of helix 12 in
ER structures may originate from the fact that the ER LBD structures were determined without the C-terminal F domain. By contrast, the biochemical data supporting the two-site model were collected using full-length ER, which includes the F domain. Only a few NR structures have been solved with sequences C-terminal to helix 12, including glucocorticoid receptor (GR; Blodsoe et al. 2002, Kauppi et al. 2003), PR (Williams & Sigler 1998), and PPARα (Xu et al. 2002). However, these NRs contain small C-terminal sequences (~10–12 amino acids or less) and are not generally considered as F domains. On the other hand, the F domains of ERα and ERβ are relatively large (41 and 30 amino acids respectively) and believed to possess conformational flexibility, as well as differences in secondary structure (Skafar & Koide 2006).

Although no structural data (X ray crystallographic or NMR) currently exist for the ER F domain, biochemical and theoretical studies have shed some light on its function (reviewed in Skafar & Zhao 2008). The most important fact related to the inconsistencies between biological full-length ER and structural ER LBD-only data is that deletion or specific mutations of the F domain eliminates the ability of tamoxifen to act as an agonist (Montano et al. 1995, Schwartz et al. 2002, Koide et al. 2007b). This key observation suggests that the structures of ligand-bound ER LBD, determined without the F domain, may not depict the precise role of helix 12 in determining the active state of full-length ER. Again, this is significant because the structural studies prior to the HT-ERβ LBD structure (Wang et al. 2006) suggest that one molecule of tamoxifen bound in the cognate ligand-binding pocket is sufficient to induce an antagonist AF-2 conformation, which is in direct conflict with biochemical studies (reviewed in Jensen & Khan 2004). Furthermore, mutation or truncation of the F domain has been shown to: alter antagonist activity, making tamoxifen a more effective antagonist (Montano et al. 1995, Schwartz et al. 2002); increase the affinity for E2 (Schwartz et al. 2002, Skafar & Koide 2006); remove a partial inhibition of dimerization (Peters & Khan 1999); alter the ability of E2 to overcome the antagonist activity of tamoxifen (Skafar & Koide 2006); alter the activity of an ERE-driven promoter in response to E2 or HT (Koide et al. 2007b); increase the degree to which ER binds coactivators, including nuclear receptor-interacting protein (RIP)-140 (Peters & Khan 1999) and SRC-1 (Koide et al. 2002); eliminate the E2-dependent activation of ERα/SP1-mediated transcription (Kim et al. 2003); and remove the protection of ligand-unbound ER from proteolysis (Tateishi et al. 2006). Interestingly, helix 12, but not the F domain, was found to be essential for ERα interactions with cytokeratins 8 and 18 (Long & Nephew 2006), suggesting an F domain-independent action that adds further complexity. Similar observations detailing the role of the F domain on the function of other NR proteins have been noted (Modarress et al. 1997, Sladek et al. 1999, Suaud et al. 1999, Ruse et al. 2002, Bertin et al. 2004, Farboud & Privalsky 2004, Petrescu et al. 2005, Takayama et al. 2007). In particular, in a study of the hepatocyte nuclear factor-4α (HNF4A), the authors assert that the characteristics of F domain-truncated mutants “may not reflect the structure, ligand-binding, ligand-conformational responsiveness and cooperativity of full-length HNF4A” (Petrescu et al. 2005).

**Evidence supporting a change from the strict use of helix 12 positioning in determining the NR agonist/antagonist state**

Considering the substantial functional complexity of the F domain, in particular the evidence that deletion of the ER F domain eliminates the agonist action of tamoxifen, a question arises as to whether the strict interpretation of helix 12 in an agonist or antagonist conformation is strictly valid for determining ER, and more generally, NR function. Observations from other studies support this notion. The agonist properties of tamoxifen were shown to be functionally and structurally distinct from those of E2, whereas its antagonist properties were different from the type II antiestrogen ICI 164 384 (McDonnell et al. 1995). Helix 12 is not observed in some crystallographic studies of ER antagonists, including ICI 164 384-ERβ LBD (Pike et al. 2001), adding to the speculation concerning its role in determining the antagonist state. In a recent structural analysis of ERα (Heldring et al. 2007a), removal of helix 12 from a LBD-only protein construct significantly enhanced the binding of corepressor peptides that specifically recognize HT-bound ER (Paige et al. 1999, Huang et al. 2002, Heldring et al. 2004). The corepressor peptides recognize a novel HT-induced binding surface, distinct from the agonist-induced AF-2 surface (Heldring et al. 2004). Furthermore, it was necessary to remove helix 12 from the protein construct in order to yield crystals of ER in complex with the corepressor peptides. It is tempting to speculate that the structural position of helix 12 was not favorable for corepressor interaction, perhaps because it was not in a conformation similar to the HT-ERβ LBD structure with a HT molecule bound in the AF-2 coactivator surface (Fig. 4F) or the GW6471–PPARA–NCOR complex (Fig. 4G).
Interestingly, a small molecule (p-chloromercuribenzoic acid) used to reduce crystal lattice motion in the study of ICI 164 384-ERβ (Pike et al. 2001) was found to bind in a similar region to the displaced helix 12 in HT-ERβ (Wang et al. 2006). It is further tempting to speculate about functional relevance of this surface with respect to helix 12, the F domain, or domain/protein interactions in general. Structures of AR LBD with small molecules bound in the AF-2 coactivator surface reveal that the sequence C-terminal to helix 12 makes β-sheet contacts to the loop region between helices 9 and 10 (Fig. 4H and I; dotted oval regions), whereas helix 12 adopts an agonist-like conformation (Estebanex-Perpiñá et al. 2007a), GR (Bledsoe et al. 2002) and PR (Williams & Sigler 1998) LBD proteins bound to agonist also make β-sheet contacts between the C-terminal helix 12 extension and the helix 9-helix 10 loop. Unfortunately, it has not been possible to obtain crystallographic structural data for the ER F domain (region C-terminal to helix 12) or the loop between helices 9 and 10. If this type of β-sheet interaction observed in the AR LBD structures is conserved in ER and other NR proteins, it suggests that helix 12 might be tethered into an agonist-like position, capping the cognate ligand-binding site, regardless of the type of ligand bound in the cognate ligand-binding site, further raising the question whether helix 12 blocks the AF-2 surface in the antagonist functional state.

A recent study of PPARG LBD (Bruning et al. 2007) tested the generally accepted model that asserts the level of NR agonism is associated with the degree to which the AF-2 coactivator surface is stabilized, namely helix 12 dynamics and positioning. Crystal structures and hydrogen exchange data obtained for six ligand-bound PPARG LBD complexes, varying from full agonists to intermediate/partial agonists, reveal that individual ligands induce unique conformational dynamics. In particular, full agonists induce a change in helix 12 dynamics, compared with ligand-free PPARG, whereas intermediate and partial agonists do not alter the dynamics of helix 12 at all. Furthermore, intermediate and partial agonists differentially alter the dynamics of the helix 2/β-sheet region. The conclusion from this study, namely that activation of PPARG is not solely determined by helix 12 positioning and dynamics, led the authors to conclude that the ‘understanding of allosteric signaling must be extended beyond the idea of a dynamic helix 12 acting as a molecular switch’ (Bruning et al. 2007). Similar observations were noted for the LBD of RXRA (Yan et al. 2004, 2006, Nahoum et al. 2007) and, more recently, ERα (Dai et al. 2008). In the case of ERα, different groups of hydrogen exchange signatures are observed for ER agonists and SERMs, suggesting that hydrogen exchange signatures can provide a method for predicting tissue-specific ligand response. Notably, the ligands display differential stabilization in helix 2, helix 2/helix 3 loop, helix 3, helix 6, the β-sheet 1/2 region, and helix 11. Moreover, two distinct groups of SERMs are noted, based on the degree of protection in the β-sheet region. Raloxifene-like molecules give rise to an intermediate degree of protection in the β-sheet region, whereas HT-like molecules afford a greater degree of protection. Of note, helix 12 displays no observable protection to hydrogen exchange upon binding any of the ligands studied, adding further uncertainty to the role of helix 12 conformational dynamics in determining the ER/NR agonist or antagonist state.

The need for an extended model of NR agonist/antagonist action is further supported by the evidence revealing the importance of a number of complex factors in determining the response to a particular ligand and the subsequent active state of the NR protein. These factors include, but are not limited to, interactions between different domains within NR proteins (Métivier et al. 2002, Glaros et al. 2006, Li et al. 2006), the notion that agonist ligands can recruit coactivator and corepressor proteins (Folkertsma et al. 2007, Gurevich et al. 2007), the observation that coregulator/transcriptional machinery interactions can influence whether tamoxifen acts as an agonist or antagonist in a particular cell environment (Smith et al. 1997, Shang & Brown 2002, Smith & O’Malley 2004, Kressler et al. 2007), NR mobility, dynamics, and degradation within the cell (Hager et al. 2004, Smith & O’Malley 2004), as well as the nature of the receptor binding to DNA (direct or indirect), including ligand dissociation after binding DNA (Anolik et al. 1996, Klinge et al. 1996a), different NR isoforms, and post-translational modifications of the receptor complex (discussed further in Wehling 1997, Chen et al. 1999, Beato & Klug 2000, Lee & Lee Kraus 2001, Nilsson et al. 2001, Steinmetz et al. 2001, Cavaillé 2002, Heinlein & Chang 2002, Stallcup et al. 2003, Perissi & Rosenfeld 2005, Duma et al. 2006, Faus & Haendler 2006, Li & Shang 2007) – all of which are not included in the functional interpretation of the LBD structural data. A recent AR LBD structural report revealed that the binding modes of coactivator and corepressor peptides are structurally similar (Jouravel et al. 2007), leading the authors to suggest that ‘the AF-2 region is utilized as a ‘communicating’ binding site for both repression and activation of AR.’ Thus, NR activation is not simply described using an ‘on/off’ switch model, where helix 12 positioning determines the degree of
agonism, but rather a complicated combination of multiple factors resulting in the final functional outcome.

Relevance to targeted ER drug development

The current approach for SERM development is afforded as a result of extensive studies pertaining to their cell type and tissue-selective function (for discussions concerning this topic, please see Diel 2002, Gustafsson 2003, Kian Tee et al. 2004, Pearce et al. 2004, Shao & Brown 2004, Smith & O’Malley 2004, Thomas et al. 2004, Knox et al. 2006, Cheskis et al. 2007). Significant progress has been made to delineate the molecular framework to target ER-specific SERMs. The bulky oxabicyclic scaffold was found to be 10–50-fold more selective for ERβ in competitive binding assays and up to 60-fold more active (Hsieh et al. 2006). Crystal structures of the LBD of ERα and ERβ in complex with these ligands reveal that two residues in particular, Met336 and Met421 in ERα or Leu384 and Ile373 in ERβ, are likely responsible for the ERβ-type selectivity. Modified versions of the ERβ-specific phytoestrogen genistein (Kuiper et al. 1998) were used to develop additional ERβ-specific ligands (Mewshaw et al. 2005), which were found to be active in two inflammation models, suggesting its use in treating chronic inflammatory diseases, including inflammatory bowel disease or rheumatoid arthritis, and later noted to be effective for treating severe sepsis (Cristofaro et al. 2006). A derivative of the phytoestrogen, 8-prenyllnaringenin, was found to be a full agonist for ERα and an antagonist for ERβ (Roelens et al. 2006), suggesting that a molecule can be developed to directly influence the opposing roles of ERα and ERβ (Hall & McDonnell 1999, Matthews & Gustafsson 2003).

Alternative methods for developing antagonist compounds to directly modulate ER-coactivator interactions have also been reported. Instead of taking the traditional approach of designing a molecule to compete for the cognate ligand-binding site, these methods aim to block NR signaling by means of direct inhibition of NR-coactivator binding. One method involves the use of peptidomimetics, which are small peptides based on the LXXLL NR recognition motif designed to bind in the AF-2 surface and inhibit ER–protein interactions (Geistlinger & Guy 2003a,b, Leduc et al. 2003, Geistlinger et al. 2004). This approach supports a wide variety of natural (amino acid-like) and non-natural side-chain scaffolds that bind to ER with relatively high affinity (nM–µM). However, the large size of these molecules precludes their use in a clinical setting. Another approach involves the development of small molecules designed to inhibit protein–protein interactions (broadly reviewed in Peczuh & Hamilton 2000, Toogood 2002, Pagliaro et al. 2004, Zhao & Chmielewski 2005). The first proof-of-principle ER-coactivator inhibitors were pyrimidine-based molecules that mimic the three leucine residues of the coactivator LXXLL peptide helix and bind to the AF-2 surface (Rodriguez et al. 2004). However, these molecules could not be studied in vivo due to their low binding affinity. More recently, it was reported that bicyclo[2.2.2]octane compounds are effective structural mimics of two key leucine residues within the LXXLL motif that bind to the hydrophobic groove of the AF-2 surface (Zhou et al. 2007). Additionally, cell- and computer-based screening methods have been used to identify novel ERα antagonist compounds that block the interaction between ERα and the coactivator SRC-3 and inhibit endogenous ERα function in MCF-7 cells (Shao et al. 2004). These compounds did not displace E2 from the ligand-binding site, but were found to bind directly to the LBD of ERα, implying that they function through the inhibition of ER-coactivator interactions. The HT-ERβ (Wang et al. 2006), PPARα (Estébanez-Perpiñá et al. 2007a), and THRB (Estébanez-Perpiñá et al. 2007b) LBD structural studies, which reveal that the small molecules indeed bind to the AF-2 surface, support the concept of small molecule modulators of NR activity. Additionally, the observation that helix 12 from a neighboring ERβ molecule in the crystal lattice associates with the second HT molecule bound in the AF-2 surface suggests that it is possible to target a SERM to the AF-2 surface with molecular properties that could recognize or fine-tune ER interaction with specific co-regulator proteins.

Concluding remarks

Numerous LBD-only structural studies have revealed key insight into the function of NR proteins and provided a platform for design of pharmaceutical modulators in the drug discovery process. However, inconsistencies between the biological and structural data highlight the fact that the ‘divide-and-conquer’ approach to studying the structure–function relationship does not always paint a complete picture that fully describes the function of the full-length, native biomacromolecule. To the best of our knowledge, no structural study of an antagonist-bound NR LBD reported to date displays a native, antagonist conformation where helix 12 occupies the AF-2 surface. We
note a few cases where non-native conditions resulted in the so-called antagonist conformation of helix 12. In addition to the aforementioned ER LBD studies (reviewed in Pike 2006), structures of Heliothis virescens ultraspirecule protein (USP) (Billas et al. 2001) and human retinoic acid receptor α/mouse RXRA heterodimer (Bourguet et al. 2000) NR LBDs resulting in an antagonist helix 12 conformation were developed using truncated F domain mutants. In the case of the mouse RXRA (Bourguet et al. 2000), an antagonist helix 12 conformation was observed only with a constitutively active LBD mutant (F318A). The authors note that the conformation of helix 12 in the F318A RXRA structure does not correlate with biological studies that reveal both native and F318A RXRA proteins bound to the coactivator TIF2. Interestingly, the authors also note that deletion of helix 12 and, therefore, its presence in the AF-2 surface is not mandatory for antagonism (Voegel et al. 1996).

Finally, a study of GR bound to the antagonist RU 486 (Kauppi et al. 2003) described three antagonist-bound structures (GR1, GR2, and GR3). In the GR2 structure, helix 12 was absent due to enterokinase cleavage. In the GR3 structure, helix 12 from a domain-swapped molecule binds to the AF-2 surface of another GR molecule between crystallographically identical subunits in the GR dimer. The structural refinement of GR1 was not finalized due to poor data, nor was it submitted to the Protein Data Bank; however, the GR1 structure was used as an example for the antagonist conformation of helix 12 in GR. One possible example of a native antagonist helix 12 conformation is found in the structure of PPARA bound to the antagonist molecule GW6471 and the NCOR peptide (Xu et al. 2002). The conformation of helix 12 in the PPARA structure is similar to the secondary interaction involving helix 12 from a neighboring molecule in the crystal lattice of the ERβ LBD structure bound to two HT molecules (Fig. 4F and G; Wang et al. 2006).

However, the sequence C-terminal to helix 12 in PPARA is short (~5 amino acids) and may allow for more flexibility in the positioning of its helix 12 compared with other NR proteins with large F domains. For many other NR proteins, no wild-type structures bound to an antagonist molecule have been reported to the best of our knowledge. Most, if not all, discussions concerning the structural basis of antagonism in these proteins, as well as efforts for structure-based development of NR antagonists, are related to antagonist ER structures as a model system. These observations, as well as increasing experimental evidence supporting a role for changes in ligand-induced conformational dynamics rather than the structural conformation of helix 12, suggest that a new model for the NR antagonist state is needed. An accurate understanding of the NR antagonist state will undoubtedly lead to improved intelligent design of NR pharmaceutical modulators.

Nature has precisely tuned the NR cognate ligand-binding site to have high affinity for its natural ligand. The studies described reveal potential for rational drug design approaches to target small molecules to functionally important surfaces, such as the AF-2 surface, which circumvents competition with the cognate ligand-binding site. Further studies are needed to address a number of issues pertaining to the relationship between the two-site antiestrogen model and the active state (agonist/antagonist activity) of ER proteins. In particular, what is the biological role of the second tamoxifen, or more generally, antiestrogen molecule in determining ER activity? What are the precise roles of helix 12, the helix 2/β-strand surface, the F domain, other domains N-terminal to the LBD, and differences in the SERM-induced conformational dynamics in regulating coregulator binding? Biochemical data suggest that antagonism induced by type I compounds arises as a result of a second, concentration-dependent ligand-binding event – is SERM agonism or antagonism dependent on the tissue-specific concentrations of these molecules? The action of type II compounds is more uncertain, as biochemical data suggest that ER can bind twice as many of these compounds as well. However, as noted above, because the extended R group portions of these molecules associate in the AF-2 surface, the binding of a single type II compound should be sufficient to induce an antagonist response.

As no structural data exist for agonist or antagonist molecules bound to a NR protein with an intact F domain, nor has the possibility for a second ligand-binding site been extensively explored, it is unclear whether the commonly accepted agonist or antagonist position of helix 12 in LBD-only protein constructs is functionally relevant. Additional structural (crystallographic or NMR spectroscopy) and functional studies are warranted to explore the role of the F domain in response to agonist and antagonist ligands, as well as its possible role in two-site tamoxifen binding to the AF-2 surface of ER. In addition to pure structural studies, the use of monobodies may provide a useful tool to study different conformations of NR proteins (Batori et al. 2002, Huang et al. 2006, Koide et al. 2007a,b). This concept is realized in a recent E2-ERα structure bound to the E2#23 FN3 monobody, where a helix structure within a loop of the monobody domain binds to the ERα AF-2 surface (Fig. 4J). Until future
studies reveal the long-awaited, three-dimensional structure of a full-length NR protein, or the LBD and F domains together, caution should be taken in the functional interpretation of LBD-only biological and structural studies.

Note added in proof

After this review was in press, three heterodimeric structures of intact PPARG and RXRA bound to: (i) agonist, partial agonist, antagonist PPARG ligands, an agonist RXRA ligand; (ii) the PPRE DNA response element and (iii) coactivator LXXLL peptides, were released in the PDB under accession codes 3DZU, 3DZY and 3E00. An article describing these structures appeared in *Nature* (Chandra et al. 2008), which represents the first intact structure reported for a NR protein. Among many new and exciting insights, the structures show that the β-sheet region of PPARG makes long-range contacts to the DNA-binding region of the RXRA. Additionally, helix 12 is observed in the active/agonist position when the NR complex is bound to a PPARG agonist, partial agonist, or antagonist ligand.


Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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