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Structural insights into selective agonist actions of tamoxifen on human Estrogen Receptor alpha

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Abstract

Tamoxifen, an anti-estrogenic ligand in breast tissues and being used as a first-line treatment in ER-positive breast cancers, is found to develop resistance followed by resumption of growth of the tumor in about 30% of cases. Whether tamoxifen starts assisting in proliferation in such cases or there exists any ligand-independent pathways to transcription is not fully understood; also, no ER α mutants have been detected so far which could lead to tamoxifen resistance. Performing in-silico conformational analysis of ER α ligand binding domain, in the absence and presence of selective agonist (Diethylstilbestrol; DES), antagonist (Faslodex; ICI), and SERM (4-hydroxy tamoxifen; 4-OHT) ligands, we elucidated ligand-responsive structural modulations of ER α -LBD dimer in their agonist and antagonist complexes and address the issue of “tamoxifen resistance”. We found DES and ICI to stabilize the dimer in their agonist and antagonist conformations, respectively. The ER α -LBD dimer without the presence of any bound ligand also leads to a stable structure in agonist conformation. However, the binding of 4-OHT to antagonist structure is found to lead to a flexible conformation allowing the protein visiting conformations populated by agonists as are evident from principal component analysis and radius of gyration plots. Further, the relaxed conformations of the 4-OHT bound protein is found to exhibit a diminished size of the co-repressor binding pocket at LBD, thus signaling a partial blockage of the co-repressor binding motif. Thus, the ability of 4-OHT bound ER α -LBD to assume flexible conformations visited by agonists and reduced co-repressor binding surface at LBD provide crucial structural insights into tamoxifen-resistance complementing our existing understanding.

Keywords

Estrogen Receptor alpha (ER α); In Silico studies; ER α transactivation; Tamoxifen resistance

Introduction

Breast cancer, accounting for more than 458,500 deaths worldwide every year, is still one of the primary malignancies in women [1]. Among several key molecular pathways, the most important one involves Estrogen Receptor alpha (ER α) [2–4] as about 70% of breast tumors

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are ER α -positive and depend on estrogenic ligand for the progression of the tumor. ER α , a member of the nuclear hormone receptor family, is a ligand-based transcription factor [5–6] that controls the expression of hundreds of genes responsible for the diverse phenotypic properties like growth, motility, and differentiation [7–8]. Among five major domains of ER α , the ligand binding domain (LBD) is considered to be the key structural domain that controls ligand-dependent regulation of ER signaling through N- and C-terminal activation function domains (AF-1 and AF-2) and as such has been the focus of intense structural studies.

Binding of estrogenic ligands (agonist) to ER α LBD brings conformational changes suitable for dimerization, recruitment of co-activator proteins and binding to the DNA estrogen response element (ERE) in the promoter region of target genes [10–11]. Similar to agonist ligands, antagonist (antiestrogenic) ligands also induce conformational changes on ER α . However, ER α conformations that result from the binding of an antiestrogenic ligand are quite different from those arising from the binding of an agonist ligand. The former is expected to prevent the binding of coactivator signal transmitting proteins, thus, acting as an antagonist, impairing hormone-dependent ER transactivation [12]. Comparison of agonist and antagonist ligand bound ER α -LBD crystal structures (3ERD.pdb and 3ERT.pdb) reveals that Helix 12 plays a critical role in agonism or antagonism. The bulky sidechain of 4-OHT provokes steric clashes with Helix 12 forcing it to adopt its characteristic antagonist conformation where it orients in the co-activator recognition groove in ER α -LBD. It is to be noted that Helix 12 itself possesses a LXXLL sequence motif that perfectly mimics the interactions with co-activator peptides, thus allowing the blockage of co-activator binding [13].

Among several classes of anti-estrogenic ligand, ICI 182,780 (Faslodex) acts as a complete antagonist in all tissues [14]. On the other hand 4-hydroxy tamoxifen is better classified as Selective Estrogen Receptor Modulator (SERM), which behaves as ER antagonists in breast tissues only [15–16]. About 30% of ER-positive breast cancers that initially respond well to tamoxifen (a Selective Estrogen Receptor Modulator or SERM) by the cessation of growth or regression, eventually resume growth despite the continued presence of the SERM and most tamoxifen-resistant tumor continue to express ER [17–18]. Recent studies have elucidated some of the molecular reasons that linked *in vitro* models of tamoxifen's estrogenic effects with clinical reports of tamoxifen resistance and is thought to originate from the various aspects of estrogen signalling, interactions with co-regulators, and the interplay with growth factor signalling pathways [19–23]. Studies on mouse model demonstrated that by blocking the co-repressor NCoR activity, 4-hydroxy tamoxifen behaved like an agonist [24]. Thus, co-repressor expression and their binding ability to the protein both could be deciding factors in tamoxifen resistance. However, the definitive molecular mechanism of tamoxifen resistance still remains unknown. To the best of our knowledge, no such structural details are available on how both the agonist and antagonist conformations of ER α are accessible in the presence of tamoxifen.

We present a structural insight into the effect of ligand selective responses on ER α transactivation pathway; we carried out four different molecular dynamics simulations of ER α -LBD dimer where in each pair of monomers is bound with two i) agonist

(Diethylstilbestrol, DES), ii) SERM (4-hydroxy tamoxifen, 4-OHT), and iii) pure antagonist (ICI 182,780, ICI) ligands. We also consider ER α -LBD dimer without any bound ligand. Our results show distinctive behaviour of ER α -LBD dimer conformational dynamics which is dependent on the bound ligand subtypes. Interestingly, ER α -LBD can form a stable dimer without binding to any ligand and in the presence of bound agonist and antagonist/SERM. DES and ICI stabilise the agonist and antagonist conformation of ER α -LBD dimer in terms of Helix 12 position. The presence of bound 4-OHT in the LBD changes the conformational dynamics of ER α -LBD dimer in such a way that both the agonist and antagonist conformations are accessible. Through in-silico simulation, we found that the antagonist conformation of ER α -LBD 4-OHT complex does allow the binding of corepressor(s) while the agonist conformation obtained from MD simulations of tamoxifen bound ER α -LBD does not allow the binding of co-repressors, since the co-repressor binding pocket is diminished. Thus, a decreased expression of co-repressor protein and/or a diminished co-repressor binding pocket might allow the ER α to switch from antagonist to agonist conformation and lead to the observed tamoxifeninduced ER α transactivation [24].

Materials and methods

Modeling of ER α homo-dimer in agonist & antagonist conformations

The crystal structure of ER α LBD homo-dimer (PDB ID: 3ERD) where each monomer is bound with an agonist ligand diethylstilbestrol (DES) has been considered as ER α LBD dimer agonist conformation. Each monomer comprises of residues 305–550 and Helix 12 is positioned properly to accommodate co-activator proteins. There are some key missing residues (residue nos. 462–469) in chain B at the dimer interface connecting Helix 8 to Helix 9 in the crystal structure (PDB ID: 3ERD). All the missing residues were modelled by using MODELLER 9.9 [25]. The missing sequence was also modelled by superimposing chain B on chain A followed by manual grafting of the missing residues from chain A to chain B using VMD [26]. Both the modelled structures were then energy minimized using GROMACS [27–28] molecular dynamics code with OPLS [29] force field and their stereochemical quality were checked PROCHECK as implemented in SAVES web server [30]. Both the agonist and antagonist dimers appear to be of very high quality; there were no residues in the disallowed region of the Ramachandran plot. One hundred percent of residues fall within the core and in extended allowed region of the Ramachandran plot. Finally, the crystal structure with manual grafting of missing residues in chain B has been used in our study as it exhibited greater symmetry of monomers in the dimer structure.

A ligand-free ER α LBD dimer was prepared from the modelled ER α LBD dimer structure by removing the bound DES from each monomer and then relaxing the resulting structure through energy minimization and equilibration at 300 K in the presence of explicit water. It is to be noted that the ligand-free ER α LBD dimer has been modelled in agonist conformation in terms of Helix 12 orientation. On the other hand, for ER α LBD dimer bound with a SERM, we used the crystal structure of ER α LBD (PDB ID 3ERT) where a SERM, 4-hydroxy-tamoxifen (4-OHT) is bound to the LBD and the Helix 12 positioned itself in such a way that prevents co-activator binding. This LBD has been used to prepare

the ER α LBD dimer in antagonist conformation using a homology modelling procedure available in the SWISS-MODEL [31] web-interface with 3ERT as a template structure.

To prepare an ER α LBD dimer bound with pure antagonist ICI 182,780 (referred as ICI throughout the text), the 3ERT crystal structure for ER α LBD has been used to dock ICI using AutoDock 4.2 [32]. All hetero atoms were deleted and non-polar hydrogens were merged as required by AutoDock. The Kollman united-atom charge model was applied to the protein. Atomic solvation parameters and fragmental volumes were added to the protein. Grid maps were generated by using the empirical free-energy scoring functions in AutoDock. A grid box of $120 \times 120 \times 120$ grid points with a grid-point spacing of $0.375 \text{ \AA}$ was considered for docking. The box was centred such that it covers the entire LBD. The 3-Dimensional co-ordinates of the ligand (ICI) were obtained from the PubChem compound library (CID 104741). Rotatable bonds were assigned and non-polar hydrogens were merged. Partial atomic charges were calculated using the Gasteiger-Marsili method for the ligand. Two hundred and fifty docking runs were performed and for each run, a maximum of 2,500,000 GA operations were carried out on a single population of 150 individuals. The weights for crossover, mutation, and elitism were default parameters of 0.8, 0.02 and 1, respectively. The lowest energy docked complex of ER α LBD monomer and ICI was selected to build the dimer based on the ER α LBD dimer bound with 4-OHT (described in the previous section) as a template. This lowest energy docked complex was then solvated and equilibrated in the presence of explicit water to prepare the system to run molecular dynamics. A schematic representation of all the three ligands considered in this study has been shown in Scheme 1.

Molecular dynamics simulation

The parameters for diethylstilbestrol (DES), 4-hydroxy tamoxifen (4-OHT), and ICI were developed according to OPLS force-field defined atomic groups. The atomic partial charges are readjusted to maintain the charge neutrality of the whole molecule. The parameters are tested by comparing the GROMACS energy minimized structures with the respective crystal structures and similar energy minimized structures obtained using CPMD [33].

Each ER α LBD dimer complex (with or without a ligand) was subjected to a preliminary short energy minimization *in vacuo* using steepest descent algorithm. Then the system was solvated with explicit water model in a cubic box with periodic boundary conditions and the box dimensions were chosen such that all the protein atoms were at a distance equal to or greater than 1 nm from the box edges. The ionization state of residues was set to be consistent with neutral pH and 12 Na⁺ ions were added to make the system neutral. The solvated system was then subjected to a second short energy minimization of 500 steps using steepest descent algorithm to eliminate any bad contacts with water. After that, a 200 ps position restrained dynamics was carried out where the complex was restrained by restraining forces while the water molecules were allowed to move. It was then followed by a 500 ps of NVT simulation at 300 K and a 500 ps of NPT simulation to achieve a suitable equilibration of the system to be simulated. Final production simulations were performed in the isothermal-isobaric (NPT) ensemble at 300 K, using an external bath with a coupling constant of 0.1 ps. The pressure was kept constant (1 bar) by using pressure coupling with

the time-constant set to 1 ps. The LINCS [34] algorithm was used to constrain the bond lengths, allowing the use of 2.0 fs time step. Van der Waals and Coulomb interactions were truncated at 1.4 nm and the GROMACS implemented SHIFT function is used to reduce the truncation error. Molecular dynamics trajectories were stored at every 5 ps.

Structural analysis were carried out by using the in-built tools of GROMACS and the secondary structure assignments were carried out with DSSP [35] module integrated with GROMACS. The RMSD matrices were computed on each of the trajectories by the least square fitting of main-chain atoms and the matrices were then processed to extract clusters of similar conformations.

Principal component analysis (PCA)

Principal Component Analysis (PCA) was performed on each of the ligand bound ER α -LBD dimer trajectories obtained from the MD simulations in order to obtain an insight into the essential dynamics of the simulated system in reduced sub-space. Mass-weighted covariance matrix of atomic positional fluctuations was calculated from each MD trajectory fitted on C α atoms of the reference structure and analyses were performed on backbone atoms. Then the essential subspace was defined in terms of the calculated eigenvectors.

Identification of Hotspot residues at the dimer interface

The essential protein-protein interface interaction of the homo-dimer was explored by using HotPoint online web-server [36] for the four MD average structures of ER α -LBD dimer (one without ligand, and three in the presence of DES, 4-OHT and ICI, respectively) obtained from covariance analysis. The server also predicts hotspot residues that are involved in crucial interactions for the dimer interface.

Binding pocket and co-repressor binding analysis

The initial and the final average structures of 4-OHT bound ER α -dimer obtained from the MD simulation were analyzed further to identify putative binding pocket using Q-10 SiteFinder [37] program. Co-repressor peptide docking at the identified binding site was carried out using RosettaDock [38] web-interfaces.

Results

In order to gain an insight into the stability and structural dynamics of ER α -LBD dimer and its dependency on the bound ligand subtypes, molecular dynamics simulations have been performed on both ligand-bound and ligand-free ER α -LBD homo-dimer. Stability of the agonist and antagonist ER α -LBD dimer conformations with respect to bound ligand subtypes has been explored using root mean square deviation (RMSD), radius of gyration (Rg), and cluster analysis. We also investigated the changes of secondary structure in the essential dimer interfaces induced by the bound ligand. A comparison of the essential dynamics of ER α dimer bound with various ligand sub-types (agonist, antagonist and SERM) has been carried out using principal component analysis. In addition, in-silico studies have been carried out to explore prototype co-repressor peptide interactions with

ER α -LBD to understand the probable mechanisms of tamoxifen resistance. Detail results are given below:

Stability of ER α dimer and its dependency on bound ligand

In Fig. 1, we exhibit the variation of dynamic parameters RMSD and R_g of ER α -LBD dimer in the absence and in the presence of three ligands (4-OHT, DES, and ICI) as a function of time.

As evident from Fig. 1A, during the first 2 ns of the simulation, all the four different systems undergo conformational readjustments according to their respective ligands and environments and monotonically reaches an equilibrium state. A closer look at each trajectory obtained from the MD simulation reveals that ER α -LBD dimers remain overall stable throughout the simulation in the presence or absence of any ligand. ER α -LBD dimers do not dissociate in the presence of a bound SERM or a pure antagonist, and remain stable throughout the simulation period. Also, interestingly, we found that ER α -LBD itself has the ability to form a stable dimer without any bound ligand. Binding of an agonist ligand, DES, in the LBD, stabilizes the dimer with an average RMSD value of 0.16 ± 0.02 nm for initial 7 ns and in last 3 ns of simulation of DES bound ER α dimer RMSD increases and stabilizes to a higher value 0.27 ± 0.17 nm. In comparison the ligand free ER α -LBD dimer where the average RMSD is 0.21 ± 0.03 nm. It is noteworthy that the binding of a SERM to the LBD induces perturbation in the dimer complex but that does not lead to any dissociation of the complex. The average RMSD of ER α -dimer when 4-OHT is bound to the LBD is 0.28 ± 0.06 nm. It is to be mentioned that the binding of ICI to the LBD, stabilizes the dimer complex further but in an antagonist conformation with an average RMSD of 0.21 ± 0.03 nm over the same simulation period. The observed high fluctuations in the RMSD profile in the case of 4-OHT bound ER α -LBD dimer signify some structural transition in the dimer complex. We provide further insight into structural changes by analysing the radius of gyration (R_g). R_g defines the overall shape and dimensions of the protein. The plot of variation of radius of gyration of each LBD dimer with time is shown in Fig. 1B. It is to be noted that for unliganded and DES-bound ER α dimer, the starting conformation for MD simulation is in agonist conformation while in case of 4-OHT and ICI bound ER α dimer, it is in antagonist conformation. Due to the orientational difference of Helix 12, the antagonist/SERM bound ER α dimer has a higher R_g compared to free or DES-bound ER α -LBD dimer. Again, it is evident from Fig. 1B that the binding of DES stabilizes the LBD dimer in agonist conformation as seen from the R_g profile which is more stable compared to the unliganded ER α dimer R_g profile throughout the course of the simulation. Binding of a pure antagonist, ICI, also stabilizes the LBD dimer but in an antagonist conformation, as evident from the stable R_g profile throughout the simulation time. On the contrary, binding of a SERM or 4-OHT displays a distinctive feature in the R_g profile. During the initial phase of the simulation, 4-OHT bound ER α -LBD dimer appears in antagonist conformation and its R_g value is comparable to that of ICI bound ER α -LBD dimer. But with the simulation over time, its R_g value decreases and converges with the R_g value of the agonist (DES) bound ER α dimer signifying a structural transition from an antagonist to an agonist conformation.

Cluster Analysis

To provide a detailed insight into all the visited conformations of ER α dimer during MD simulation, RMSD matrices have been computed and results are displayed in Fig. 2. In absence of any ligand, ER α -LBD dimer is quite stable throughout the simulation period. There is a single population of the dimer conformation observed during the simulation which is structurally very close to the initial agonist conformation in terms of Helix 12 orientation (Fig. 2A). Binding of an estrogenic ligand, DES, also stabilizes the agonist conformation. Throughout the MD simulation, the initial agonist conformation is highly populated and structurally distinct conformational clusters are very less frequently visited (Fig. 2B). The RMSD matrix profile is distinctively different when 4-OHT, a SERM, is bound in the LBD. It is to be noted that the initial structure of ER α -LBD dimer in the MD simulation is in antagonist conformation. Throughout the simulation period, this initial antagonist conformation is much less populated and structurally distinct conformations are more frequently visited. In fact, in the R_g plot (Fig. 1B), we observed that when 4-OHT is bound in the LBD, with time the ER α -LBD dimer shifts from an antagonist conformation to an agonist one. So, the distinct conformational subspace is shape wise very close to the agonist conformation. While for pure the antagonist ICI, the starting structure of ER α -LBD dimer for MD simulation is in antagonist conformation with respect to Helix 12 orientation and throughout the simulation period this antagonist conformation is highly populated. This signifies that the binding of pure antagonist, ICI, shifts the agonist-antagonist conformational equilibrium towards the antagonist structure.

Effect of bound ligand sub-types on the essential ER α dimer interface

We further studied the effect of bound ligand sub-types on the dimer interface of ER α -LBD dimer. Essential dimer interface has been characterized from the changes in solvent accessible surface area (SASA) of the monomer and dimerized ER α -LBD. Upon dimerization, those residues, that undergo a reduction in the SASA value $> 20 \text{ \AA}^2$, have been considered to be involved in the dimer interface. As evident from Fig. 3, four regions mainly contribute to the dimer interface. Residues 421–437 of Helix 7 are defined as region I (red), residues 448–461 from C-terminal portion of Helix 8 as region II (orange), residues 473–490 from Helix 9 (magenta) as region III and residues 473–490 from Helix 10 and N-terminal portion of Helix 11 (blue) as region IV. In addition, we also consider Helix 12 (cyan), defines as region V. It is worth mentioning that in DES bound ER α -LBD dimer, Helix 12 orients towards the dimer interface but in the 4-OHT or ICI bound ER α -LBD dimer, Helix 12 orients away from the dimer interface, thus losing its contribution from the essential dimer interface.

We have then analysed the effect of bound ligand on the secondary structure of each region of the dimer interface. Region I, II and III adopt a stable α -helical structure and the binding of any agonist/antagonist ligand does not induce any perturbation in the secondary structural profile of these regions (data not shown). Region IV, on the other hand, has been involved in crucial interactions in dimer interface and undergoes significant changes on its secondary structure upon ligand binding, as shown in Fig. 4A.

An interesting observation is that in the absence of a bound ligand, Helix 10 and 11 appears as a continuous helix. It is worth mentioning that in other nuclear receptor family proteins, helix 10 and 11 are separated by a kink or bend. As evident from the Fig. 4A (II), the binding of an estrogenic ligand, DES, induces strong turn propensity in between Helix 10 and 11 and separates the two helices (indicated by the yellow line in the middle of the region). In the presence of 4-OHT (SERM), the turn propensity of the region between helix 10 and 11 reduces further. Interestingly in the last 4 ns of simulation period, the turn propensity increases compared to the initial 3 ns. On the contrary, when ICI, a pure antagonist, is bound to the LBD, the turn propensity of the intermediate region between helix 10 and 11 diminishes and the two helices appear as a continuous helix. This observation plays an important role in co-repressor protein recognition (discussed in section ***“In silico modelling and analysis of co-repressor binding: Implication to tamoxifen resistance”***).

Fig. 4B displays the variation in the secondary structural profile of Helix 12 with simulation time. In the absence of any ligand or in the presence of bound DES, this region adopts α -helical structure throughout the simulation time. However, the secondary structural profile undergoes a significant distortion in the presence of 4-OHT. During the simulation, Helix 12 mostly appears as a helical turn. MD simulation reveals that the binding of ICI in the LBD completely disrupts the secondary structure of this region; it appears as highly flexible bend/coil like conformation.

Interaction energy and “Hotspot” residue analysis

In Table 1, we summarize the interaction energies between each of the four regions with the other monomer to explore the energetic consequences of the bound ligand sub-types. Without the presence of any ligand bound to the LBD, the main energetic contributions come from region III and IV. The Coulomb interaction dominates for region III while both the Coulomb and the LJ (Lennard-Jones) interactions play a dominant role in the recognition of region IV. In the presence of the bound estrogenic ligand DES, the Coulomb interaction between region I and the other LBD monomer reduces significantly. However, the interaction profile of region II remains unaltered. Interestingly, the interactions between region III and the other LBD monomer reduce significantly while region IV interacts more strongly. For both the regions, significant changes are observed in the Coulombic interactions. When DES is bound to the LBD, maximum contribution to the interaction energy comes from region IV [39]. Binding of 4-OHT and/or ICI induces a different pattern in the interaction energy profile. In the presence of 4-OHT in the LBD, the energetic contribution of region I increases while it decreases significantly for region III. Again, the main energetic contribution comes from region IV. In the case of 4-OHT bound to ER α dimer, the interaction profile of region IV is comparable to the interaction energy profiles of ligand free ER α -LBD dimer with a minor reduction in the LJ interaction energy. On the contrary, in the presence of ICI, the interaction energies between two dimers reduce in general and again the main interaction energy contribution comes from region IV. For region III, the Coulomb interaction energy increases significantly compared to 4-OHT bound ER α -LBD dimer.

In accordance with the interaction analysis, it has been observed that when 4-OHT and ICI is bound to the LBD, the C-terminal end residues of region I and IV do not involve in the dimer interface. We observed a leucine zipper interactions between two monomer involving Leucine 508 and 509 from Helix 10 (Fig. S1, supplementary material). These interactions form the core of the dimer recognition that remain unperturbed in the presence of any bound ligand, whether agonist or SERM, or antagonist. Analysis of the critical residues involved in the dimer interface in the presence various ligands have been summarized in Table 2. It is to be noted that Threonine 483, Alanine 505, Leucine 508, Leucine 509, and Arginine 515 are necessary and important residues at the dimer interface and their contributions remain unchanged in the presence of bound agonist/antagonist. Tyrosine 459 acts as a crucial hotspot residue at the dimer interface only when DES or 4-OHT or no ligand is present in the LBD, but it losses contribution as a hotspot residue when pure antagonist ICI is bound to LBD. Likewise, Asparagine 455 and Arginine 515 in chain B play a dominant role in dimer recognition in the presence of bound ICI in LBD but in the presence of bound 4-OHT and DES, these residues lose their contribution from dimer interface recognition.

Principal Component Analysis

We then carried out principal component analysis (PCA) to identify most significant structural dynamics from MD trajectories. Mass-weighted covariance analysis has been performed on the MD trajectories and the essential subspace has been defined in terms of eigenvalues relative to the corresponding eigenvectors. First few components are more informative as is evident with their high eigenvalues while the latter components carry very little information about the dimer dynamics (Fig. 5A). This feature is evident for both ligand-free and ligand-bound ER α -LBD dimer.

As evident from Fig. 5A, in the presence of DES and 4-OHT, the eigenvalue of the first principal component (PC1) is much higher than the ligand free or ICI bound ER α -LBD dimer. In all the four cases, the first two principal components (PC1 and PC2) together contribute the most to the essential subspace. A bi-dimensional projection of PC1 and PC2 is presented in Fig. 4B. It is evident from the figure that, in all the four cases, the PC1 varies more broadly compared to the PC2. This corroborates well to the eigenvalue distribution plot where PC1 has the highest eigenvalue (Fig. 5A). Interestingly, the PC1 vs PC2 representation reveals that for 4-OHT bound ER α dimer, the 2-D projections are much wider compared to the other three cases. This indicates that the 4-OHT bound ER α -LBD dimer structure access more diverse conformational space during the MD simulation.

In silico modelling and analysis of co-repressor binding: Implication to tamoxifen resistance

An important question is how the agonist and antagonist conformational distributions generated from molecular dynamic simulation of ER α -LBD dimers in the presence of agonist, antagonist, and SERM ligands can be used to interpret the classical clinical fallacy of “Tamoxifen resistance”. It is to be noted that selective conformations of 4-OHT bound ER α dimer generated by the MD simulation is structurally more closer to the agonist bound dimer complex but its Helix 12 is still in antagonist position, i.e., bound to the co-activator binding groove. This observation implies that co-activators might not play the decisive role

to explain the resistance phenomenon as its binding surface is occupied by Helix 12. This shifts our focus to co-repressor proteins. However, this is to be noted that, the co-repressor proteins are extremely large ones and are expected to have several contacts with ER α including its DNA Binding Domain (DBD), as reported by Varlakhanova *et. al.* [40]. Importantly, co-repressor proteins share a binding motif LXXXIXXXL which is very similar to LXXLL, the common binding motif for activator proteins. Recent structural studies indicate that LXXXIXXXL motif also binds to the AF2 co-regulator interacting surface of ER α [41] obtained by deleting Helix-12. But in the agonist like conformation of 4-OHT bound ER α dimer obtained from our MD simulation, this surface is also blocked by Helix-12. This raises the possibility that the co-repressor might have an alternate binding affinity to the usual Helix 12 binding site formed by Helices 11, 4/5 and 3. In fact, Hu *et. al.* [42] reported that the CoRNR2 co-activator peptide binds to the usual Helix 12 binding site formed by Helices 11, 4/5 and 3 in agonist bound RXR protein (retinoid X receptor, another nuclear hormone protein). To explore this unique mode of co-repressor binding possibility to the ER α homo-dimer, we further characterize the usual Helix 12 binding pocket or new co-repressor binding pocket framed by Helices 11, 4/5 and 3 for both agonist and antagonist ER α conformations in presence of bound 4-OHT. We referred this mode of co-repressor recognition as “non-classical” mode. Interestingly, the Q-site-finder, a protein’s binding pocket finding algorithm, also predict a possible binding pocket in this non-classical co-repressor binding site.

In the 4-OHT bound initial antagonist conformation of ER α , the non-classical corepressor binding site has a volume of 231 Å³ (Fig 6). It is to be noted that in this conformation, Helix 10 is nearly in continuation with Helix 11 and the linker loop of Helix 11 and 12 goes round enough to frame the co-repressor binding site. On the other hand, the agonist like ER α conformations with bound 4-OHT appeared in the later stages of simulation reveals that Helix 11 is slightly tilted and the kink between Helix 10 and Helix 11 is more prominent (Fig. 4A, III). This tilted position of Helix 11 allows further coiling of the loop regions between Helix 11 and Helix 12. Due to the concomitant movement of Helix 11 and the loop regions joining Helix 11 and Helix 12, the non-classical co-repressor binding site further reduces to 115 Å³. This nearly diminishes the co-repressor binding possibility. Co-repressor peptide binding in this site was performed by RosettaDock program. In 4-OHT bound ER α antagonist conformation, the peptide binds successfully in this non-classical co-repressor binding site, as is evident from Fig. 7 (deep green). But the docking failed for 4-OHT bound ER α agonist conformation generated through MD simulation. We aligned the MD average ER α agonist conformation on the co-repressor bound ER α agonist conformation. From Fig. 7B, it is clearly evident that there are steric clashes between the side chains of the co-repressor peptide and the linker loop of Helix 11 and 12. The loss of the co-repressor recognition in 4-OHT bound ER α agonist conformation might induces some transcriptional activity of the dimer complex. Nevertheless, this site of co-repressor recognition is a low affinity site, since Helix 12 and its linker loop region provides steric constraints in co-repressor recognition at this site. Binding of Helix 12 in the co-activator/co-repressor binding site raises the possibility of co-repressor peptide binding at this non-classical site of LBD and presents a possibility of agonist action by 4-OHT bound ER α and explains the 'tamoxifen resistance'.

Discussions

As the formation of ER α dimer is one of the essential steps that regulate DNA recognition and gene expression, we aim the present work to provide a detailed structural insight on the effect of ligand-regulated response on ER α -LBD dimer using in-silico studies. We simulate ER α -LBD dimer in the presence of a bound agonist (Diethylstilbestrol, DES), a SERM (4-hydroxy tamoxifen, 4-OHT), and a pure antagonist (ICI 162,571, ICI) and elucidate the conformational changes and the dimer interface to understand the ligand-selective response of ER α . ER α is found to have the intrinsic ability to form a stable dimer without any ligand which is in accordance with the kinetic study that shows unliganded ER α exists as stable dimers with a slow dissociation rate [43]. The presence of an agonist ligand, DES, in the LBD stabilizes the dimer further while the binding of 4-OHT induces a structural fluctuations in the dimer complex but that does not lead to any destabilization of the dimer which is again in agreement with the solution study [43]. The presence of ICI in the LBD, also allows ER α to exist as a dimer complex in an antagonist conformation. For the dimer interface, we found that the essential dimer surface comprises of residues 421–437 of Helix 7, residues 448–461 from C-terminal portion of Helix 8, residues 473–490 from Helix 9, residues 496–523 from Helix 10 and Helix 11; and those remain nearly unchanged with agonist, antagonist or SERM. Leucine 508 and 509 residues are involved in a Leucine zipper interaction that forms the core of the dimer recognition. Secondary structure analysis of the MD trajectories reveals a distinctive feature. Estrogenic ligand DES stabilizes the α -helical structure of the dimer interface region while 4-OHT and ICI induces structural destabilisation, particularly on Helix 12. In the presence of 4-OHT at LBD dimer, the α -helical content of those interface regions decrease with a concomitant increase in turn propensity. However, ICI, a pure antagonist, exhibited strong ability to disrupt the secondary structure of the interface region and Helix 12. This is consistent with the fact that in the only crystal structure of rat ER β LBD with pure antiestrogen ICI 164,384, the longer side chain of ICI 164,384 binds along the co-activator recruitment site. This abolishes the interaction of helix 12 with the LBD such that it is completely disordered. This overall conformation could favour the recruitment of co-repressors and resemble misfolded conformation of ER α , thus promoting the degradation of the protein [44].

Principal component analysis (PCA) and cluster analysis on MD trajectories reveal an interesting observation. PCA reveals that in the essential subspace, 4-OHT induced ER α conformational dynamics are quite distinct from a pure antagonist induced structural dynamics. This observation can be used to interpret the observed estrogenic effects of tamoxifen on the endometrium, bone, and cardiovascular system [16], and also for the tamoxifen-resistant tumor growth in breast tissues. These observations can be rationalized by the fact that the antagonist action of tamoxifen is mainly attributed to its effect on Helix 12 and is highly dependent on absolute and relative level of co-repressor and co-activator proteins expressed in particular tissues. We further studied the possibility of a prototype co-repressor peptide binding in a distinct binding site formed by Helices 11, 4/5 and 3, as depicted in RAR-RXR heterodimer complex [42]. Our *in silico* simulation data suggests that the recruitment of proper co-repressor stabilizes the antagonist conformation of ER α dimer, thus deactivating DNA recognition and gene transcription. But the absence of co-repressor

in the “non-classical” binding cleft shifts the equilibrium of the dimer complex towards the agonist conformation, thus promoting ER α transactivation. These effects might be dominant in bone and endometrium tissues. Tamoxifen resistance in breast tissues can also be rationalized in terms of altered relative expression level of co-repressor and co-activator proteins. A decreased expression of co-repressor protein can stabilize the agonist conformation of ER α dimer paving the way for any tamoxifen-induced ER α transactivation. In cellular perspective, tamoxifen resistance represents a complex phenomenon due to the involvement of various co-regulators [24,45] and their correlated expression profiles together with the involvement of various signalling pathways [20–22]. Studying ligand-selective structural modulations of these agonist and antagonist conformations of LBD, we were able to identify structural differences pertaining to “tamoxifen resistance” which complement the clinical observations regarding the role of co-regulatory proteins [24]. On therapeutic perspectives, the identification of a possible new co-repressor binding site and its role on antagonist to agonist conformational transition provides an alternate therapeutic strategy to design peptide or peptidomimetic drugs that can be used to overcome the resistance to tamoxifen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. & B. Variations in RMSD and radius of gyration (R_g) of ER α dimer with time. Black line represents ER α dimer without the presence of any bound ligand, while red, green and blue lines represent ER α dimer in the presence of DES, 4-OHT and ICI, respectively.

Figure 2. RMSD matrices of ER α dimer computed from MD trajectory. A: Unliganded ER α dimer; B, C & D represent ER α dimer in the presence of bound DES, 4-OHT and ICI, respectively.

Figure 3. ER α LBD dimer. Essential dimer interface has been coloured differently. Residues 421–437 from each monomer coloured red, residues 448–461 coloured as orange, residues 473–490 coloured as magenta, residues 496–523 coloured as blue and Helix 12 has been coloured as cyan.

Figure 4.

Secondary structure profile with simulation time. A. residues 496–523 from Helix 10 and N-terminal portion of Helix 11 (region IV) B. Helix 12 region. I: Apo ERα dimer; II, III & IV represent ERα dimer in presence of bound DES, 4-OHT and ICI, respectively.

Figure 5.

A. Normalized Eigenvalue distribution for first 10 Eigen vectors obtained from MD trajectory. B: 2-D projection of first two principal components (1 & 2) for ligand free and bound ER α dimer. Red colour represents ER α dimer without presence of any bound ligand, while green, blue and cyan colour represents ER α dimer in presence of bound DES, 4-OHT and ICI, respectively.

Figure 6. Predicted “non-classical” co-activator binding pocket using QSiteFinder. Pocket volume is rendered as red. A. Antagonist ERα structure in presence of bound 4-OHT (starting structure of MD simulation). B. Agonist like ERα conformation in presence of bound 4-OHT observed ~ 7 ns of MD simulation.

Figure 7.

A. Docked structure of co-repressor peptide (Red) bound ERα LBD antagonist conformation (green). B. Manual alignment of ERα agonist conformation (cyan) with bound 4-OHT. The side chains in the co-repressor binding side are shown in stick representation.

Scheme 1.

Chemical structures of the three ER α ligands used in this study. A: Diethylstilbestrol B: 4-Hydroxytamoxifen C: ICI 182,780.

Table 1

Comparisons of average Coulomb and LJ interaction energies (kJ/mol) of the four regions of one monomer with the other monomer LBD involved in dimer interface.

Ligand Name	Region I		Region II		Region III		Region IV	
	Coulomb KJ/mol	LJ KJ/mol	Coulomb KJ/mol	LJ KJ/mol	Coulomb KJ/mol	LJ KJ/mol	Coulomb KJ/mol	LJ KJ/mol
No Ligand	-81.0	-45.8	-80.5	-89.3	-327.7	-68.4	-202.5	-341.9
DES	-21.1	-66.2	-79.3	-73.6	-117.8	-54.8	-295.8	-351.3
4-OHT	-171.8	-76.2	-47.3	-91.5	-61.6	-78.2	-208.4	-317.5
ICI	14.63	-28.42	-61.5	-91.1	-141.10	-52.2	-193.9	-290.94

List of “Hotspot” residues of ER α dimer with their estimated potential. Residues those are specific for a particular bound ligand have been designated in bold and estimated high potency values (cut-off 30) for dimer interactions are also in bold.

Table 2

Residues	Potential	Residues	Potential	Residues	Potential	Residues	Potential
No ligand		DES		4-OHT		ICI	
Y459(A)	18.53	N455(A)	17	N455(A)	20.14	M427(A)	18.7
T483(A)	18.66	Y459(A)	18.72	Y459(A)	18.53	T460(A)	19.25
L 497(A)	17.37	T483(A)	19.5	T483(A)	18.66	H476(A)	18.98
L504(A)	44.75	H501(A)	21.45	Q500(A)	17.67	T483(A)	18.66
A505(A)	17.44	R505(A)	17.44	L504(A)	37.59	Q500(A)	17.67
L508(A)	45.10	L508(A)	37.94	A505(A)	17.44	L504(A)	37.59
L509(A)	34.72	L509(A)	34.72	L508(A)	45.1	A505(A)	20.73
H 513(A)	21.98	L511(A)	22.38	L509(A)	27.56	L508(A)	45.1
R 515(A)	26.08	R515(A)	19.33	H513(A)	19.41	L509(A)	34.72
N455 B	19.76	T431(B)	19.44	R 515(A)	23.9	R 515(A)	22.84
Y 459 B	22.31	R455(B)	20.14	H516(A)	17.69	H516(A)	18.78
H 476 B	19.83	Y459(B)	20.69	M427(B)	21.49	M427(B)	18.16
T 483 B	18.66	T483(B)	22.1	V458(B)	20.34	R455(B)	19.67
L 504 B	37.59	Q500(B)	17.67	L479(B)	28.57	Y459(B)	20.4
A 505(B)	20.73	R505(B)	17.44	T483(B)	19.5	T483(B)	22.94
L508(B)	52.26	L508(B)	45.10	R505(B)	17.44	R505(B)	17.44
L 509(B)	34.72	L509(B)	34.72	L508(B)	45.1	L508(B)	45.1
L 511(B)	22.38	L511(B)	22.38	L509(B)	34.72	L509(B)	34.72
R 515(B)	18.65	H513(B)	21.41	H513(B)	21.41	H513(B)	21.47
H 516(B)	18.75			R515(B)	25.88	R515(B)	23.86
				H516(B)	18.78		