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Original Article

Characterization of ligand type of estrogen receptor by MD simulation and mm-PBSA free energy analysis

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Abstract: Estrogen receptor is a transcription regulator and can bind structurally distinct ligands with full agonistic, SERMs, or full antagonistic properties. Crystal structures of the ER ligand binding domain (LBD)-complexed with full agonists or SERMs show that these ligands induce two different orientations of Helix12 in LBD and generate two different conformations, agonist conformation (A conformation) and AF2 antagonist conformation (B conformation). To understand how ER ligands interact with LBD structurally and energetically, we docked 3 full agonists, 9 SERMs and 2 full antagonists in both the A and B conformation of ERα LBD and performed a 4-step molecular dynamics (MD) simulation on all 28 complexes followed by mm-PBSA binding free energy calculation. We found that all full agonists prefer the A conformation while all SERMs prefer the B conformation. Analysis of the mm-PBSA energies revealed that calculated total binding free energies (delta PBTOT) and the difference of VDW between complex and the sum of receptor of ligands and ligand (delta VDW) have the order of full agonists>SERMs>full antagonists. However, the PB surface term has the order of full antagonists>SERMs>full agonists. We also found that the sum of the RMSD of mainchain atoms of Helix12 and all atoms of ligands in the A conformation is significantly lower for full agonists than that of the other ligands. Together, we conclude that the three types of ER ligands interact with the A and B conformations of ERα LBD differently and same type of ligands interact simlarly. These findings will be useful in understanding the mechanism of ER antagonism and can be used in ligand type prediction.

Keywords: Estrogen receptor, antagonism, full agonist, full antagonist, SERM, agonist conformation, AF2 antagonist conformation.

Introduction

Estrogens are lipophilic hormones produced by ovaries and adrenal glands. Estrogens exert a wide variety of physiological effects on cell growth, proliferation and differentiation via estrogen receptor (ER), which belongs to the nuclear receptor (NR) superfamily. The NR family members share a conserved structural architecture consisting of six structural domains A through F. There is one transactivation region each in the AB structural domain at the N-terminus (AF1) and in the F structural domain at the C-terminus (AF2). In between, there is a DNA binding domain, a hinge region and a ligand binding domain located at the C, D and E structural domain, respectively.

Estrogen receptor is a transcription regulator [1]. When ER binds to its natural ligand, 17β-estradiol (EST), it dissociates with its co-repressor protein and homo-dimerizes. It then binds to DNA element called estrogen receptor element (ERE) and recruits co-activators and the transcriptional machinery and helps to initiate transcription of its regulated genes. Over expression of ERα or over activation of ER mediated transcription is involved in the pathogenesis of breast cancer and ovarian cancer [2, 3].

ER has an effect on a broad array of tissues [4-8]. It regulates the development of reproductive system, bone metabolism, and maintains cardiovascular and central nervous systems. Many structurally distinct chemicals have also been shown to bind to ER with one of the three properties as full agonists that only activate ER upon binding; full antagonists that are capable of blocking ER activation; and SERM (selective estrogen receptor modulators) that functions as agonists in some tissues and cell types, but as antagonists in others. Crystal structures of ER

Table 1. The name, PDB code, antagonism type, binding mode and experimentally determined binding affinity (in kcal/mol) of selected ER ligands

Ligand Name	PDB code	Antagonism type	Binding mode	ΔG_{exp}
EST	1GWR	Full agonist	A	-13.73
DES	3ERD	Full agonist	A	-14.22
THC	1L2I	Full agonist	A	-11.5
OHT	3ERT	SERM	B	-14.27
RAL	1ERR	SERM	B	-13.49
AEJ	1XQC	SERM	B	-13.19
PTI	1UOM	SERM	B	-12.48
AIH	1XP1	SERM	B	-14.28
AIU	1XP6	SERM	B	-15.78
AIJ	1XP9	SERM	B	-13.73
AIT	1XPC	SERM	B	-13.55
GW5 (GW-5638)	1R5K	SERM	B	-11.92
AOE (ICI 164,384)	1HJ1	Full antagonist	C	-6.78
FAS (ICI 182,780)	Modelled	Full antagonist	C	-10.31

LBD in complex with different ligands have been solved. For example, ERα LBD in complex with full agonist diethylstilbestrol (DES) and a peptide derived from ER coactivatior shows that Helix12 is accurately positioned and is in contact with both ligand and the peptide [9]. The crystal structure of LBD complexed with 4-OH-tamoxifen (OHT) revealed that OHT is located in the same binding site as that of DES [9]. However, Helix12 is pushed away due to the bulky size of OHT with a 130° shift. As a result, Helix12 occupies the binding pocket of the GRIP1 peptide. Therefore, OHT binding induces a different LBD conformation that blocks LBD coactivator recruitment. These two different conformations are named agonist and AF2 antagonist conformation and will be referred as the A and B conformation in this study, respectively. In addition, the structure of ERβ LBD in complex with full antagonist, ICI 164,384 (AOE), shows that Helix12 was prevented from adopting either the A and B conformations and as a result, the helix is highly mobile and cannot be precisely located in the crystal structure [10].

To understand how ER ligands interact with ERα LBD A and B conformations and how their interactions are related to their antagonism activity, we modeled 14 ER ligands, which consists of 3 full agonists, 9 SERMs and 2 full antagonists, into both A and B conformations of ERα LBD and performed 1-ns Molecular Dynamics (MD) simulations. Binding free energies were computed by mm-PBSA method and structural analyses were performed. We found that all ligands can bind to both conformations, although it is energetically more favorable for ligand to bind in their native mode. Calculated total binding free energies, some energy terms, and RMSD all cluster by ligand types. The three types of ER ligands interact with the A and B conformations of ERα LBD differently and same type of ligands interact simlarly. Therefore, the ligand type of a potential ligand for ER may be predicted by the way it interacts with the A and B conformation of ERα LBD.

Experimental methods

Model building

Crystal structures of ERα LBD complexed with 3 full agonists and 9 SERM ligands were collected from RCSB protein databank (Table 1). Examination of these structure complexes revealed that the three agonist complexes all adopt an agonist conformation (A conformation) and the nine SERM complexes are all in the AF2 antagonist conformation (B conformation). Because structures are similar and consistent across each series, only one structure is chosen for each A and B conformation. Crystal structure of ERα LBD complexed with DES (pdb code 3ERD) was chosen for A conformation and ERα LBD complexed with OHT (pdb code 3ERT) was chosen for B conformation. All the rest of ligands were docked into these two A and B models by structural alignment of their corresponding crystal structure with these two model structures.

ERα LBD complexed with full antagonist was not available in the RCSB databank at the time of

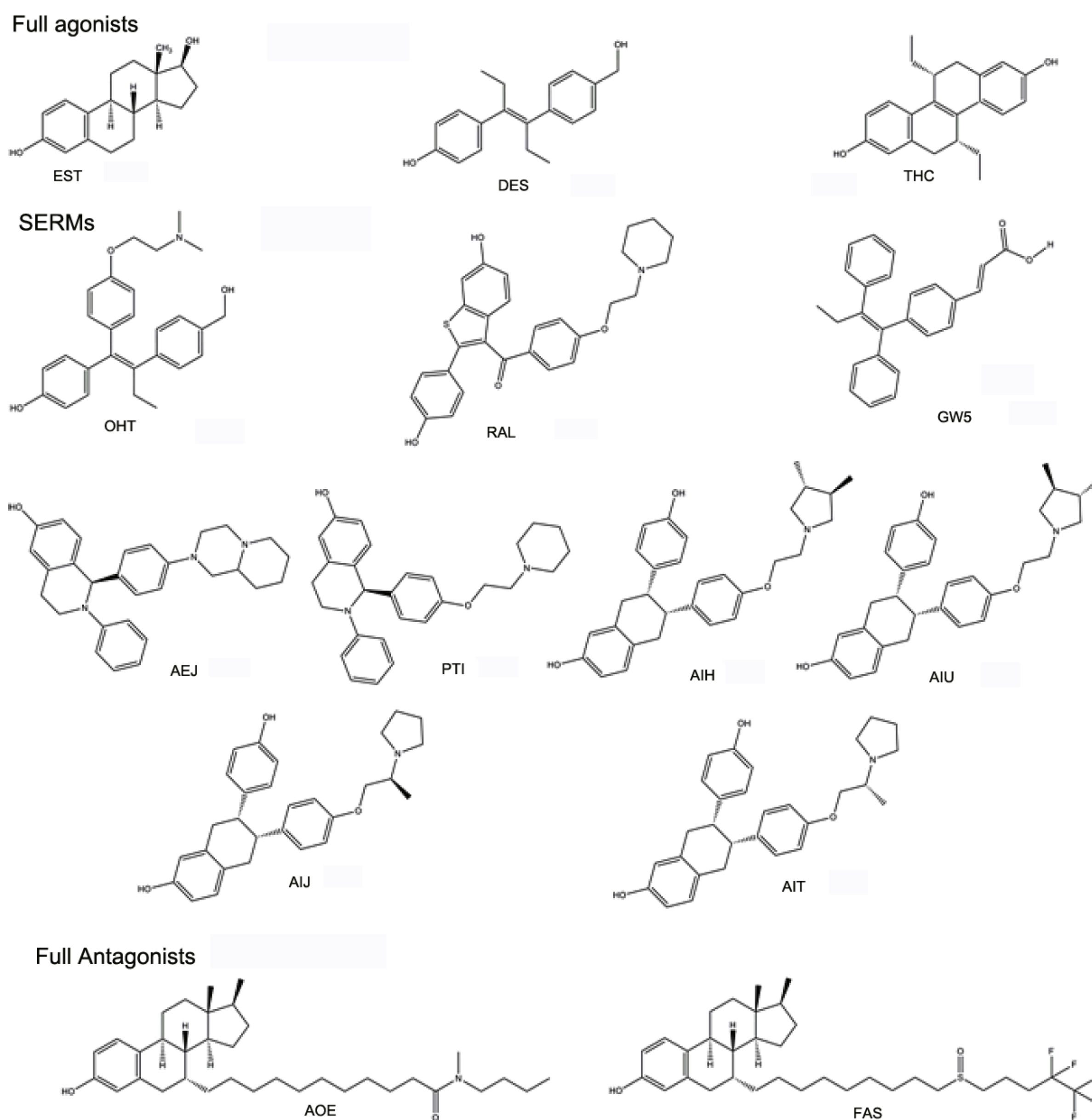

Figure 1. Chemical structures of selected ER ligands in this study.

searching. However, ERβ LBD complexed with full antagonist AOE was available (**Table 1**). The overall structure and ligand binding pocket of ERβ LBD complexed with full antagonist AOE is similar to that of the ERα LBD A and B mode except that Helix12 cannot be located. Consequently, AOE was modeled into ERα LBD A amd B conformation by structural alignment between ERα and ERβ LBD. Lastly, another full antagonist of ERα, FAS, was modeled into ERα LBD A and B conformations guided by the AOE structure complex. A Total of 14 ER ligands were selected to run simulation in the A and B mode ER LBD (**Figure 1**). The chemical structures of the 14 selected ligands are shown in **Figure 1**. Some minor adjustments to certain side chains of residues were made as needed to remove apparent collisions and clashes after structure alignments.

Ligand parameterization, MD simulations and data analysis

For all the 14 ligands, electrostatic potentials

were calculated by GAUSSIAN98 at the HF/6-31G* level of theory. Partial charges were fitted by RESP method of the antechamber module of AMBER9 and GAFF force field parameters were assigned by tleap of AMBER9. FF03 parameters and hydrogen atoms were assigned to the receptor A and B mode by tleap module of AMBER9.

MD simulations of the 28 complexes were carried out using the AMBER9 package. All agonist complexes were solvated in a 70 x 71 x 69 Å box of water and antagonist complexes in a 70x73x75 Å box of water, respectively. Appropriate number of counter ions was added to neutralize each system. Particle Mesh Ewald (PME) was employed to calculate the long-range electrostatic interactions and the nonbonded cutoff was set to 8.0 Å.

Each system was equilibrated by a four step protocol prior to production MD. First, the solvated structure complexes were minimized with a restraint of 500kcal/mol-$Å^2$ applied to the protein complex. The restraint was then removed and the energy of the protein complex was minimized. In these two steps, 500 and 1000 steps of steepest descent minimization followed by 500 and 1500 steps of conjugate gradient minimization were applied, respectively. Each system was then heated up from 0 K to 300 K gradually over a timescale of about 20 ps controlled by Langevin temperature equilibration scheme with a collision frequency of 1.0 ps^{-1}. Then the system was equilibrated at 300K for 100ps using constant volume periodic boundaries. Week restraint to the protein complexes and SHAKE procedure to all bonds containing hydrogen atoms was applied to each system in the above steps. Restraint to the protein was removed in the final 5ns equilibrium run. The production MD simulation of 1ns employed the same condition of the final equilibration step.

One hundred snapshots were collected from the production trajectory for mm-PBSA free energy calculations. The structures of the ligand and receptor are extracted from the snapshots of the complex. The binding free energies were then computed by taking the difference between the mm-PBSA free energy of the complex and that of summation of the ligand and receptor. The electrostatic contribution to the solvation free energy was determined by mm-PBSA in AMBER9. Linear approximation was used to solve the Poisson-Boltzmann equation. Grid size was set up as 0.5 Å, and the dielectric constant for the solute and solvent were set to 1 and 80, respectively. The values of bondi atomic radii were taken from AMBER9 except that of the fluorine atoms which were missing in AMBER9 in molecules FAS were set as 1.5 Å. The structures with lowest total potential energies for all 28 trajectories were generated using the ptraj module of AMBER9. These structures were compared with the original input structures and root mean square deviations (RMSD) were calculated by rms of AMBER9. Two–tailed student T-tests were applied to the means of calculated energies and RMSD values of different ligand types.

Results and discussion

Molecular modeling of 28 ERα LBD-ligand complexes

Crystal structures of ERα LBD-complexed with 3 full agonists [9, 11], 9 SERMs [9, 12-15] and 3 full antagonists [10, 16] were collected (Table 1 and **Figure 1**). These ligands were docked into two model structures of ERα LBD: the agonist conformation (A conformation) and the AF2 antagonist conformation (B conformation) as described in Experimental Methods. As a result, altogether 28 structure models of ERα LBD complexed with 14 different ligands were built.

Calculated binding free energies are in good agreement with crystal structures

We calculated the binding free energies by mm-PBSA method. The binding free energy is computed by taking the difference between the mm-PBSA free energy of the complex with that of the ligand and receptor: $\Delta G_{bind}=G_{complex}-G_{receptor}-G_{ligand}$, where $G=G^{solute}+G^{solvent}$. The G^{solute} term for each system can be obtained by $G^{solute}=E-TS$. E represents an average of energies obtained from MD simulation and is the sum of the following 3 terms: “non-bonded electrostatic energy + 1,4-electrostatic energy” (ELE); “non-bonded van der Waals energy + 1,4-van der Waals energy” (VDW) and “bond, angle, dihedral energies” (INT). S is the entropy contribution that is omitted from our calculation. The $G^{solvent}$ term can be obtained by $G^{solvent}=G^{es}+G^{nes}$. G^{es} is the electrostatic contribution which is obtained by the PB method, and G^{nes} (PBSUR) is the non-

Table 2. Calculated energies (kcal/mol) of ligands in A and B conformations

	Conformation A			Conformation B		
		DELTA			DELTA	
LIGAND	LIGAND PBSUR	VDW	PBTOT	LIGAND PBSUR	VDW	PBTOT
EST	3.5	-39.76	-30.5	3.51	-38.22	-28.72
DES	3.7	-38.45	-31.28	3.69	-40.13	-26.81
THC	3.98	-47.19	-34.99	4.01	-41.87	-33.77
OHT	5.15	-52.52	-29.36	5.1	-47.69	-39.9
RAL	5.64	-60.86	-40.59	5.58	-56.83	-42.38
GW5	4.56	-50.41	-36.79	5.74	-48.63	-37.04
AEJ	5.36	-59.9	-39.13	5.8	-54.78	-43.35
PTI	5.48	-62.92	-40.34	4.57	-55.45	-43.04
AIH	5.71	-66.63	-33.29	5.52	-55.8	-39.59
AIU	5.79	-61.74	-35.53	5.55	-57.36	-45.07
AU	5.51	-60.19	-41.97	5.31	-57.61	-45.09
AIT	5.54	-56.87	-39.21	5.35	-57.64	-40.49
AOE	6.93	-78.23	-61.07	6.95	-75.88	-63.37
FAS	7	-77.84	-63.45	6.9	-78.02	-50.73

electrostatic contribution and is proportional to the solvent-accessible surface area of the molecule. As the entropy term is not included in this study, the calculated free energy (PBTOT) is literally only composed of E and $G^{solvent}$ terms.

Although our simulation time is short and we omitted the time consuming entropy calculations, the calculated binding free energies are in good agreement with crystal structures in that all ligands have more favorable calculated binding free energies in their native mode. The calculated binding energies indicate that it is energetically favorable for each ligand to bind to ERα LBD in both A and B mode (Table 2). However, all three full agonists have a more favorable binding free energies in the A conformation and all nine SERMs in the B conformation. This is consistent with what have been observed in crystal structure complexes. For the two full antagonists, the more preferred conformation is A and B for AOE and FAS, respectively. Crystal structure of ERβ LBD-AOE complex shows that Helix12 is disordered, highly mobile, and cannot be located in a crystal structure. Therefore, neither A nor B conformation is the dominant conformation of these two complexes. Nevertheless, it is possible that AOE and FAS have different preferences over these two conformations.

Our calculated binding energies support the equilibrium theory of ERα LBD. Ligands modeled in both A and B conformations have favorable binding free energies, but with preference for one conformation over the other [17]. It is possible that Helix12 in the unliganded ERα LBD is highly flexible and it constantly transits between the active A conformation and the inactive B conformation. This finding is consistent with the observation that unliganded ER has some basal transcriptional activity [18]. Some ER co-activators may favor the A conformation. ER full agonist favors and stabilizes the A conformation itself with or without the surrounding co-activators and activates ER. ER SERMs may bind to ERα LBD in both A and B conformations, but more preferable in the B conformation. Co-activators in different tissues may function and help SERMs work as either agonist or antagonist. Full antagonists, such as ICI 164,384 (AOE) and ICI 182,780 (FAS), do not stabilize either A or B confirmation and ER Helix12 remains flexible similar to the unliganded form.

Total binding free energies cluster by ligand type

Although almost all selected ligands binds to ER with high affinity as experimentally determined (Table 2), the calculated binding free energies cluster by their ligand type with an order of full agonists>SERMs>full antagonists (Figure 2A). When the ligands are modeled in the A conformation, the mean of total binding free energy of full agonists (-32.26 kcal/mol), is significantly higher than that of SERMs (-37.36 kcal/mol) which is significantly higher than that of full antagonists (-62.26 kcal/mol). When ligands are

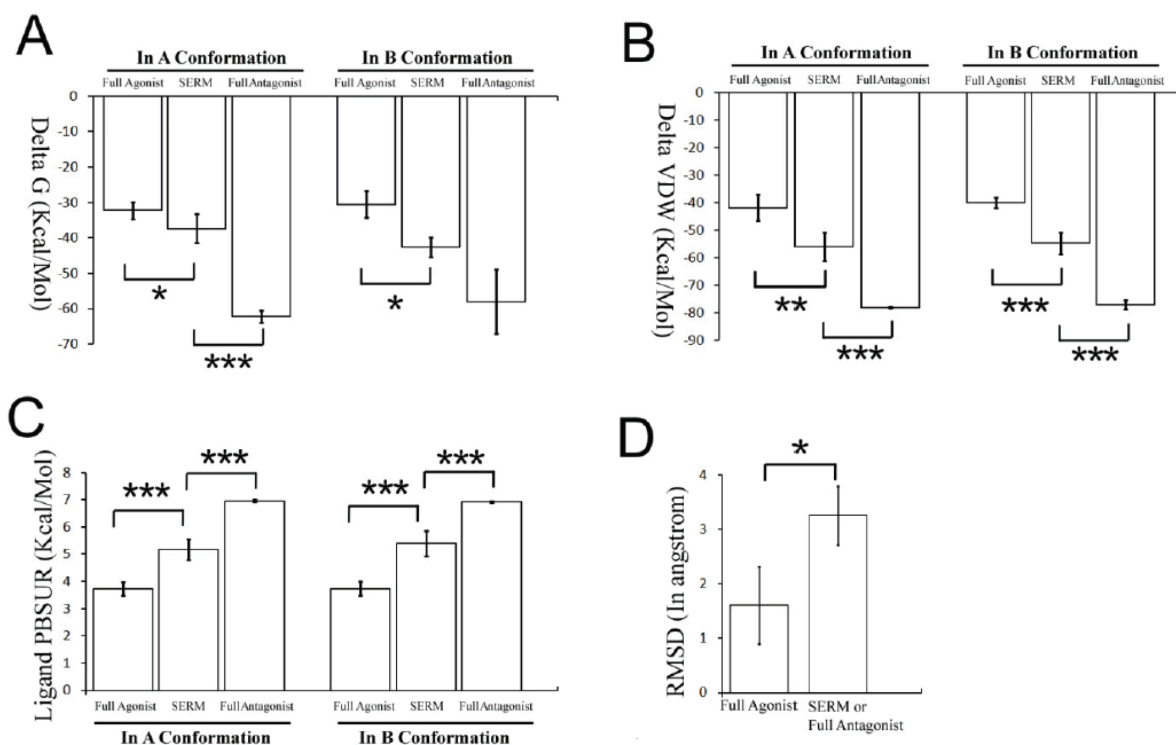

Figure 2. Mean and standard deviation of calculated energies and RMSD of full agonists, SERMs and full antagonists. A. Mean and standard deviation of calculated total binding free energy in both conformations. B. Mean and standard deviation of delta VDW energy term in both conformations. C. Mean and standard deviation of PBSUR of ligands in both conformations. D. The sum of RMSD of Helix12 and ligand when ligand is modeled in A conformation. *$p<0.05$, **$p<0.01$; ***$p<0.001$ (two tailed T-test).

modeled in the B conformation, the mean of total calculated binding free energy of agonists (-29.77 kcal/mol) is significantly higher than that of SERMs (-41.72 kcal/mol). However, no significant difference was found between the mean of total calculated binding free energy of SERMs (-41.77 kcal/mol) and that of full antagonists (-57.05 kcal/mol), although there is a ~15 kcal/mol difference between the two. This insignificant difference could be due to the small sample size of the full antagonists used in this study.

The fact that the calculated total binding free energies cluster by ligand type is reasonable and reflects the chemical properties of the ligands. Compared with full agonists, SERMs are not as stiff, which may result in a larger configurational entropy loss upon ligand binding. In addition, SERMs are larger in size which likely result in higher entropy loss with the protein due to the larger cavity created in ERα LBD by SERMs. The two full antagonists, AOE and FAS, are even larger in size and more flexible than SERMs due to the long acyl side chain (Figure 1) and, thus, may generate largest entropy loss which we omit in our calculations. Therefore, the ranking of the calculated binding free energies from high to low in the order of full agonists, SERMs and full antagonists may be resulted from the chemical nature of the ligands and may serve as predictor for the type of an ER ligand.

Calculated energy terms cluster by ligand type

The energy terms of all 28 simulations were collected and analyzed. We found that some energy terms also cluster by ligand types. First, the delta VDW energy term cluster with an order of full agonists>SERMs>full antagonists in either A or B conformation (Table 2, Figure 2B). When ligands are modeled in A conformation, the delta VDW energies are -38.45 to -47.19 kcal/mol for full agonists, -50.41 to -66.63 kcal/mol for SERMs and -77.84 to -78.23 kcal/

Table 3. RMSD of helix 12 and ligand in A conformation of ERα LBD.

LIGANDS	RMSD OF LIGAND(Å)	RMSD OF HELIX12(Å)	RMSD SUM(Å)
EST	0.13	0.68	0.81
DES	1.14	1.05	2.19
THC	0.71	1.08	1.79
OHT	1.51	1.01	2.52
RAL	2.23	1.23	3.46
GW5	0.59	2.60	3.19
AEJ	2.23	1.28	3.51
PTI	2.18	0.95	3.12
AIH	1.39	2.93	4.32
AIU	1.65	0.83	2.49
AIJ	2.27	1.28	3.55
AIT	1.34	1.93	3.26
AOE	1.58	1.11	2.69
FAS	1.70	1.97	3.67

mol for full antagonists. The mean of the delta VDW of agonists is significantly higher than that of SERMs, which in turn is statistically higher than that of full antagonists. When ligands are modeled in B conformation, the delta VDW energies are -38.22 to -41.87 kcal/mol for agonists, -57.64 and -66.63 kcal/mol for SERMs and -75.88 to -78.02 kcal/mol for full antagonists, respectively. The mean of the delta VDW of agonists in B mode is significantly higher than that of SERMs, which in turn is significantly higher than that of full antagonists.

VDW interaction is non-specific interaction. The difference of delta VDW term may reflect the size of the interacting ligands. Larger ligands that have more atoms have more favorable VDW interactions if no clashing occurs. The statistically significant difference of the means of full agonists, SERMs and full antagonists may suggest that the method we used can successfully detect the size difference of the three types of ligands and thereby cluster them.

Second, the values of the ligand PBSUR term cluster by ligand types, but in a reversed order with full agonists<SERMs<full antagonists (Table 2, Figure 2C). Each ligand has similar PBSUR value in both A and B conformations. In the A conformation, the mean of full agonists (3.72 kcal/mol) is significantly lower than the mean of SERMs (5.18 kcal/mol) which is significantly lower than that of full antagonists (6.97 kcal/mol). Similarly when ligands are modeled in the B conformation, the mean of full agonists (3.73 kcal/mol) is significantly lower than the mean of SERMs (5.39 kcal/mol) which in turn is significantly lower than that of full antagonists (6.93 kcal/mol).

The PBSUR term is the nonelectrostatic contribution of solvation effects and is proportional to the solvent-accessible surface area of the molecule. It is noteworthy that it is not the difference of PBSUR term (delta PBSUR) but the PBSUR term of ligand that have significant difference among these three types of ligands. The statistically significant difference of the means of the three types of ligands suggests that full agonists in general have less solvent accessible area than SERMs and full antagonists.

RMSDs of ligand and Helix12 cluster by ligand types

The structure of the frame with lowest total potential energy was computed for each trajectory and RMSDs to the initial A and B structure models were calculated. RMSDs of main chain atoms of residues 532 to 550 which consists of Helix12 and the loop on the N terminal end of Helix12 to the initial A and B conformations are reported in Table 3 along with RMSDs of all atoms of the ligand and the sum of the two types of RMSD values.

As shown in Figure 2D, the RMSD of Helix12 and RMSD of ligands of full agonists in the A

conformation were all below 1.5 angstrom. However, for all other ligands including SERMs and full antagonists, the RMSD of Helix12 or RMSD of ligand or both RMSD values are higher than 1.5 Å. As a result, the sum of the two RMSD values in full agonists is lower than 2.2 angstroms, but higher than 2.5 angstroms in the rest of ligands. Correspondingly, the mean of the RMSD sum of full agonists (1.60 Å) is significantly lower than that of the non-agonists (SERMs and full antagonists, 3.25 Å).

The value of RMSD sum in A conformation reflects how compatible a ligand is in the A conformation. The A conformation has a more stringent structure compared with the B conformation due to the smaller binding pocket. When full agonists are modeled in the A conformation, little adjustment is required for the ligant to be compatible in ERα LBD. When SERMs or full antagonists are modeled in the A conformation, however, larger movements are required to avoid clashing and unfavorable interactions, which generate larger RMSD. Therefore, the RMSD sum is a good structure feature that can distinguish full agonists from SERMs and antagonists.

In summary, we have shown that ER ligands cluster by their functionality in calculated total binding free energies, delta VDW and ligand PBSUR when modeled in either A or B conformation of ERα LBD. Our findings suggest that full agonists, SERMs and full antagonists interact with the A or B conformation of ERα LBD differently. The antagonistic property of a given ligand can be detected by the way they interact with the two conformations of ERα LBD via MD simulation and mm-PBSA energy analysis albeit the small sample size of this study and, thus, we may be able to predict the functionality of any ER ligand using this method. In addition, it is possible when larger number of ligands are introduced, more features such as hydrogen bonding occupancy and conformations of certain amino acid residues can be revealed to be useful features as well. Tissue specificity predication of SERMs may also be possible by investigating larger number of SERMs with available tissue specificity data.

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