Investigation of Newly Designed Human Estrogen Receptor Inhibitors from Benzophenones Derivatives (BPs) by Molecular Docking and Molecular Dynamic Simulation

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ABSTRACT

Background: Hydroxylated benzophenones exhibited estrogenic activity in human breast cancer cell, but their activities varied markedly. Previous studies showed that some benzophenones (BPs) and their hydroxylated derivatives behaved as weak estrogens (E2) in the environment. However, the available reported data is insufficient to describe structure-modification relationship of these molecules that can be developed as potential breast cancer drug. Objective: The present study aims to design and propose new morpholine ether BP (1-4c) and p-benzoyl-L-phenylalanine derivatives (1-4b). The molecular docking approach was carried out to study the potential interactions between newly designed BPs with human estrogen receptor α (hERα) and to predict the active sites and the correct binding geometries for each ligand. In addition, a 10-ns molecular dynamics simulation was conducted to further explore the stability and the dynamics behavior of the hERα-4c complex. Results: Our docking results show that the hydroxyl derivative of benzophenone morpholine ether 4c exhibited lowest binding affinity ΔG, towards the protein estrogen receptor with the highest cluster docked in the same orientation compared to the p-benzoyl-L-phenylalanine derivatives. To determine the stability between hydroxyl derivative of benzophenone morpholine ether 4c and apo conformation of hERα, we performed a 10 ns molecular dynamics simulation using AMBER FF99SB force field available in Gromacs 5.0.7, sampling the conformational changes of Helix 12 upon binding with 4c. The overall results from the simulation of the complex did not show any transition between an unfolded conformation to either an agonist or an antagonist state. MD analysis of the hERα-4c complex system reported a 0.39 ± 0.06 nm root mean square deviation values. RMSF profiles of the complex system also show the flexibility of residues 330-340, 460-470, 498 and 525-544. Our results indicate that amino acids Arg394, Glu353, Thr347, Asp351, Lys529, Gly521 and His524 participate in the hydrogen bonds network throughout the simulation. Conclusion: This study lays the groundwork for studying the interactions of the new estrogenic BPs with hERα and to assess the estrogenicity of new BPs which will be synthesized. Based on the molecular docking and molecular dynamics simulation, we found that ligand 4c has the potential for application as inhibitor for breast cancer cell.

INTRODUCTION

Breast cancer is a type of cancer which originates in the tissues of the breast, mostly from the inner lining of the lobules or the milk ducts which supply milk (Izumori et al., 2010). This kind of cancer responds to hormonal therapy and the presence of human estrogen receptor α (hERα). Estrogen receptor is associated with a more favorable response and short-term prognosis (Hunt, 1994).

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The estrogen receptors (ERs) are steroid receptors located in the cytoplasm and on the nuclear membrane and they are a member of the nuclear receptor (NR) superfamily (Germain et al., 2003). They regulate an enormity of processes starting in early life and continue through sexual reproduction, development and end of life (Hui et al., 2014; Paris et al., 2008). ER is a ligand-activated transcription factor that has important functions in many tissues and plays a critical role in the etiology of breast cancer (Hunt, 1994; Couse and Koroach, 1999; McGuire, 1978). Because ER is an important target for breast cancer treatment and prevention, numerous molecules and herbs have been evaluated for their binding towards ER and elicit their inhibitory activity (Gong et al., 2016; Telang et al., 2016). More than 20 different 3D protein structures have been solved of the hERα LBD of NRs and divided into at least to three different structural conformations. The major difference between the three conformations of the ERα LBD are the position of Helix12 (H12), which confers the specific activity; an apo conformation (Tannenbaum et al., 1998), where H12 is extending away from the core of the LBD, an agonist conformation (Warnmark et al., 2002), which becomes the transcriptional active conformation of the protein upon the binding of co-activators, H12 closed over the binding site which is called a “mouse trap”, and finally an antagonist conformation with H12 resting in the co-activator binding pocket (Shiau et al., 1998). Frativ (2015) have proposed an ERα activation-deactivation pathway as well as the sequence of the binding events during receptor activity modulation. The author reported that H12 in ERα switched from apo to agonist or even antagonist conformation and presumed that the conformational change during MD simulation was related to stabilization-destabilization of the substructural parts of the receptor.

Estrogens such as 17β-Estradiol (Figure 1a), are one among the five steroidal hormones occur naturally as female reproductive hormone. Even before the hormone estrogen is used up by the body, it has to bind to the estrogen receptor proteins. Breast cancers tend to be sensitive to estrogens which will enhance the growth of the cancerous cells with estrogen receptor on their surface are called estrogen receptor-positive cancer or ER-positive cancer (Taioli et al., 2010; Maynadier et al., 2008).

Benzophenone (BP), an aromatic ketone or diphenyl ketone, is a well known and an important intermediate in the preparation of many commercial and industrial materials such as cosmetics and medicines. Ongoing research and clinical trials provide ample evidence that BP-compounds possess diverse pharmacological potencies which include anti-inflammatory (Chen et al., 2009; Venu et al., 2007), antimalarial (Kohring et al., 2008), antianaphylactic (Neves et al., 2007), antitubercular (Chen et al., 2008), antiviral (Ferris et al., 2005), antiandrogenic (Suzuki et al., 2005), antimitotic (Liou et al., 2002), anti-cancer (Wang et al., 2009; Zabillalaa et al., 2016) properties in vitro and in vivo. The effects of 10 BPs on the proliferation of breast cancer cells and on structurally different ER-target gene transcription in MCF-7 breast cancer cells have been investigated (Kerdivel et al., 2013). The docking experiments for the BPs with ligand binding pocket of ERα highlighted the necessity of a hydroxyl group to permit proper interaction of the BPs in the ERα ligand-binding pocket. Moreover, the study have shown the interactions of BPs with the ERα is not similar to the interactions between ERα with β-estradiol, as BPs do not interact directly with His524, but only form H-bond interaction with Arg394 and Glu353 and π-π interaction with Phe404 (Kerdivel et al., 2013).

Estrogenic and anti-estrogenic activity as well as ERα and ERβ selectivity of some BP derivatives have been reported (Kunz et al., 2006; Molina-Molina et al., 2008; Cosnefroy et al., 2012). The results have shown that the estrogenic potencies of these compounds are much lower compared to β-estradiol or the potent pharmaceutical estrogen, ethinylestradiol. Competitive ER binding assays have shown that BPs compete with β-estradiol binding at the ER ligand binding site. Although the findings confirmed the relatively low affinity of BPs for ERs, which was estimated to be 100-1,000 times lower than that of the natural estrogens, the analysis suggested the direct actions of BPs via ERs. Similarly, Akahori and the co-workers (2005) have reported multilinear regression (MLR) analysis of the quantitative prediction of binding affinity followed by docking simulation in order to predict the binding potency of alkylphenols, phthalates, diphenylethanes and benzophenones to human ERs and found that the binding of BPs and phthalates to the estrogen receptor (ER) are weak and in a narrow range. On the other hand, docking simulations of imidazolylmethylibenzophenones showed high inhibition with respect to aromatase active site (Gobbi et al., 2007). Thus, the assessment of estrogenic potencies of BPs in breast cancer cells in relation to the ER interaction requires further analysis.

The present study aims to design and propose new morpholine ether BPs (1-4c) and p-benzoyl-L-phenylalanine derivatives (1-4b, Figure 1. The molecular docking was carried out to predict the active sites and the correct binding geometries for each ligand and the results were compared to β-estradiol. In addition, a 10-ns molecular dynamics simulation was conducted to further explore the stability and the dynamics behavior of the hERα dimer-4c complex as well as to investigate the flexibility of apo ERα Helix 12 (H12) conformational change in dimer form to reproduce the H12 transformation from an unfolded conformation to an agonist or an antagonist form. The molecular dynamics simulation of hERα forming complex with the newly designed morpholine ether BP was performed to determine the stability and to describe the transition path of the extended H12 in the PDB: 1A52 structure. It is expected that such findings will provide helpful insights into the design of novel and selective estrogen receptor modulators (SERMs) molecules.
Preparation of Protein – Ligand structures:

The three-dimensional structural coordinates for hERα-LBD dimer was obtained from the Protein Data Bank with the ID code 1A52. This apo structure was employed to study the H12 transformation from an unfolded conformation to an agonist or an antagonist form with the newly designed morpholine ether BPs. Initial coordinates for estradiol and all water molecules were deleted. The protein structure was energy-minimized using steepest descent algorithm method for 200 steps to remove bad contacts using GROMACS, version 5.0.7. The optimized ERα structure was then used as receptor in all docking process. The input structures for the ligands were generated and optimized using a semi empirical PM3 method (Stewart, 1989) available in Gaussian 03 software (Frisch et al., 2004). The resulting ligand coordinate files were saved in PDBQT format in AutoDockTools.

Molecular docking:

The docking studies of the ligands with the protein have been carried out using AutoDock 4.2 software (Morris et al., 1998; Morris et al., 2009). Grid maps were calculated using AutoGrid 4 and AutoDockTools based on the coordinates of estradiol in the crystal structure (PDB: 1A52). Grid maps were centred on x, y, and z coordinates (107.27, 13.94, 96.38) with the default grid box size of 70 × 70 × 65 Å and grid spacing of 0.375 Å for chain A, while chain B in the homo dimer ERα LBD was empty.

The initial ligand position, orientation and dihedral offset were set as random. The number of torsional degrees of freedom for each ligand was determined using AutoDockTools. Docking was conducted using the Lamarckian genetic algorithm. Each docked compound was derived from 100 independent docking runs that were set to terminate after a maximum of 25 × 10^6 energy evaluations, the population size was set to use 300 randomly placed individual. The best and the lowest binding energy conformation between ERα-BPs as well as ERα-estradiol was selected to be analyzed using Chimera (http://www.cgl.ucsf.edu/chimera) (Pettersen et al., 2004), LigPlot (Wallace et al., 1995).

Molecular dynamics simulation:

The Gromacs 5.0.7 program (Berendsen et al., 1995; Lindahl et al., 2001) with the AMBER FF99SB force field (Lindorff-Larsen et al., 2010; Shaw et al., 2010; Yang et al., 2006) was used for the simulation. The topology of 4c structure was generated using ACPYPE available in the Amber-Tools package. The atomic coordinates of the protein-ligand was obtained from the lowest binding energy which corresponds to the most stable conformation from the previous hERα-4c complex docking calculation. The TIP3P model (Mark and Nilson, 2001) was used for the water molecules in a cubic box volume with 1.2 nm spacing distance around the surface of the system. To neutralize the system, counter ions were added to balance the charge of the protein. The complex was minimized using steepest descent method for 6000 steps to remove bad contacts. The complex was first equilibrated for 200-ps MD equilibration under NVT condition with restraints on the protein. A 200-ps MD equilibration under NPT condition at 300 K and 1 bar were then carried out to ensure the whole system was at equilibrium. Finally, a 10 ns MD simulation was carried out at 1 bar and 300 K. The cutoff radii for Coulomb and van der Waals interactions were set to 12 Å and were updated every 2 fs. The particle mesh Ewald (Darden et al., 1993; Essmann et al., 1995) method was used for correcting electrostatic interaction. The LINCSD (Hess et al., 1997; Hess and Lincs, 2008) algorithm was used to constrain the bonds with hydrogen atoms. The temperature and pressure were kept at 300 K and 1 atm. A Berendsen thermostat and Parrinello–Rahman barostat were used to maintain the constant temperature and pressure, respectively.
RESULTS AND DISCUSSION

Docking Studies:

Molecular docking experiments were conducted for β-estradiol, (1-4)c and (1-4)b, Figure 1, against the X-ray crystal structure of β-estradiol-bound hERα receptor ligand binding domain (ERα-LBD, PDB Code 1A52) (Tanenbaum et al., 1998) using AutoDock 4.2.6 software (Morris et al., 1998). To evaluate the validity of the docking system, the bound substrate, β-estradiol, was removed from the crystallized structure of ERα, PDB: 1A52 and re-docked to the estrogen receptor. Results indicate that the X-ray crystallography conformer was identical to the docked conformer. Moreover, the superimposed binding pose of estradiol shown in (Figure 2a) are similar to those reported previously for both X-ray crystallographic structure and docking homology model (Tanenbaum et al., 1998; Katarina et al., 2013).

Binding pose with the lowest docked energy belonging to the top-ranked cluster was selected as the final model for post-docking analysis with AutoDock Tools (Morris et al., 1998; Morris et al., 2009), Chimera (Pettersen et al., 2004) and LigPlot (Wallace et al., 1995). The free energies of binding (ΔGb) and calculated inhibition constants (Ki) using AutoDock are summarized in Table 1. The docking poses for β-estradiol displayed a single mode of ligand-receptor interaction, with the binding energy of -9.67 kcal/mol, which is in agreement with the previous study by Katarina et al. (2013). On the other hand, the docking poses for other ligands display multiple frequency and the most populated ones with the lowest energy in each derivatives, 4b and 4c, were selected for further investigation.

The first group of BP is composed of p-benzoyl-L-phenylalanine and its derivatives, (1-4)b, Figure 1. Table 1 shows the hydroxyl derivatives of benzophenone morpholine ether (1-4)c, display multiple clusters docking conformation. It is observed that the hydroxyl derivative of p-benzoyl-L-phenylalanine 4b displays highest cluster of 91 conformations docked in the same orientation. The phenolic ring B on 4b forms contacts that are similar to that of ring A on β-estradiol with human ERα sharing residues Glu353 and Arg394 through hydrogen-
Fig. 3: Schematic diagrams of the protein-ligand interactions using Ligplot. (a) Estradiol E2, (b) hydroxyl derivative of \( \beta \)-benzoyl-L-phenylalanine \( 4b \), and (c) hydroxyl derivative benzophenone morpholine ether \( 4c \).

Table 1: The approximate free energies of binding (\( \Delta G_b \)) and inhibition constants (\( K_i \)) calculated using AutoDock.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Binding Energy (( \Delta G_b )) (kcal/mol)</th>
<th>Inhibition constant (( K_i )) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)( \beta )-Estradiol</td>
<td>-9.67</td>
<td>81.36</td>
</tr>
<tr>
<td>1b</td>
<td>-9.38</td>
<td>134.20</td>
</tr>
<tr>
<td>2b</td>
<td>-9.00</td>
<td>254.74</td>
</tr>
<tr>
<td>3b</td>
<td>-9.44</td>
<td>120.97</td>
</tr>
<tr>
<td>4b</td>
<td>-9.46</td>
<td>116.93</td>
</tr>
<tr>
<td>1c</td>
<td>-10.83</td>
<td>11.51</td>
</tr>
<tr>
<td>2c</td>
<td>-10.60</td>
<td>17.06</td>
</tr>
<tr>
<td>3c</td>
<td>-11.14</td>
<td>6.77</td>
</tr>
<tr>
<td>4c</td>
<td>-11.35</td>
<td>4.80</td>
</tr>
</tbody>
</table>

bond interactions. This is in keeping with the previous reports on the interaction of aromatic group in BPs insecticides and bisphenol (Celik et al., 2008; Baker et al., 2012). The authors reported that the interaction took placed in the cavity in close proximity with Glu353 and Arg394. Meanwhile, the tail from alanine group which
is in contact with ring A in 4b (Figure 2b) is engaged in hydrophobic interaction with the ER binding site as well as making hydrogen bond formations with Asp351 and Lys529, Figure 3b.

The hydrophobic and hydrogen bond formations following the interactions between the BP derivatives with human ERα are summarized in Table 2. The most populated cluster of hERα-4c has the lowest binding energy of -11.35 kcal/mol and engages more predominantly in hydrophobic interactions with ERα binding site compared to 4b (Figure 3c). This can be attributed to the fact that 4c grabs onto ER-LBD with two “hands” through the morpholine and hydroxyl groups thus forming stronger interactions compared to just one hydroxyl group for 4b. The alanine group which forms contact to ring A in 4c (Figure 2c) takes part in both hydrophobic interactions and hydrogen bond formations with the backbone amine group of Lys529 and with carboxylic acid of Thr347. Besides, the phenolic ring B on 4c forms hydrogen-bond interaction with Glu353.

Comparing the two derivatives of BPs (1-4)h,c, we found that the core BP molecule with the addition of alanine group on ring A is essential for a maximum estrogenic activity because it takes part in both hydrophobic interaction and hydrogen bond formation with the backbone amine group of Lys529 and with carboxylic acid of Asp351 and Thr347 for all derivatives. The hydroxyl group on ring B in 4b is ideal because it allows the BP derivatives to interact with the Arg394 and Glu353 thus enhancing the stability between the benzo phenones and hERα and confirm the observations made by previous researchers (Kerdivel et al., 2013). Meanwhile, 4c with the two “hands” through the morpholine and hydroxyl groups, takes part in both hydrophobic interactions and hydrogen bond formations and this results in lower binding energy.

**Molecular dynamics simulation:**

MD simulations were performed for the hERα-4c complex and the RMSD and radius of gyration (Rg) of the complex during the simulation were recorded in Figure 4. The RMSD value of backbone atoms over simulation time is 0.39 ± 0.06 nm, Figure 4a. It can be seen that the RMSD value increases for hERα-4c complex system until 5000 ps before it was slightly reduced and the value remains constant for the rest of the simulation time. The initial large variation is expected to occur due to the orientational difference of H12 in both chains and this may indicate unstable simulations of the apo complex. However, it is not surprising and it is also not the first time such high RMSD value especially for the apo estrogen receptor model was observed (Ng, 2016). Recently, Celik and co-workers (2007) reported that the apo model of estrogen receptor was more dynamic and has the RMSD values between 0.2-0.5 nm in comparison to the antagonist model which has the RMSDs values between 0.2-0.4 nm while the agonist model has lower values ie between 0.18-0.2 nm.

Radius of gyration, Rg, defines the overall shape and dimensions of the protein by calculating the mass-weighted root mean square distance of a collection of atoms from their common center of mass. The plot of the variation of radius of gyration of the hERα-4c complex with time is shown in Figure 4b. The Rg values for hERα-4c complex system started to stabilize around 8000 ps and remains so until the end of simulation, indicating that the equilibrium has been achieved. Initially the Rg value for the hERα-4c complex system was 2.4 nm and then stabilized at 2.35 ± 0.01 nm.

To understand the structural basis for the observed differences in RMSD fluctuations between the 4c ligand bound apo hERα-LBD conformation during MD simulation, we have analyzed the RMSF (root mean square fluctuations) per residue to identify the regions of high fluctuations and the results are summarized in Figure 5a. The high RMSD fluctuations of the 4c bound to the hERα are mainly due to the long loop region between Helix 8, Helix 9 and N-terminal region of Helix 9 (from residues 460 to 470). In contrast, H12 has been found to be highly flexible with the fluctuation involving residues 525 to 544.

The average LJ interaction energy during the simulation is shown in Figure 5b, it can be seen that the average LJ energy is -225 kJ/mol along the simulation time which increases slightly at around 5400 ps. The analysis of the hydrogen bonds formation of hERα-4c complex during the last 1000 ps is shown in Figure 6. The results revealed that 4c forms three hydrogen bonds with Arg394, Glu353 and Thr347, which may contribute to the enhance stability of the complex. It was previously shown by Celik et al. (2008), that BPs such as insecticides DDT and its metabolites bind particularly well to hERα LBD. They tend to bind in one or two
different orientations with both the phenyl rings pointing towards Glu353 and Arg394 in agreement with the hERα-4c complex formation.

![Fig. 4: Molecular dynamics simulations results for (a) The RMSD of all backbone atoms of hERα-4c complex. (b) The RMSF of all backbone atoms of ERα-4c complex during 10 ns.](image)

![Fig. 5: Analysis of molecular dynamics simulations (a) RMSF profile of all backbone atoms of hERα-4c complex (b) LJ energy variation of apo hERα-4c complex.](image)

![Fig. 6: Snapshot of apo hERα-4c complex at 9 ns showing the hydrogen bond formation (blue lines) of 4c with Arg394, Glu353 and Thr347.](image)

The MD simulation was conducted in order to obtain more information about the dynamic behavior of H12 in dimer hERα with a single 4c. The results shown in Figure 7 reveal that H12 in chain B, ie the monomer
with the ligand, forms stable conformation starting from 3 ns until the end of the simulation time. Meanwhile, H12 in chain A is more dynamic but no transition from the unfolded conformation to either an agonist or an antagonist state was observed. It is anticipated that longer simulation time may produce a slightly different results and the study is currently in progress.

![Fig. 7: Snapshots of the conformational dynamics of dimer hERα with single ligand, 4c complex during 10 ns simulation. The X-ray structure (PDB ID 1A52) for chain A is shown in green, chain B in cyan, H12 in red for both chains and the 4c ligand in magenta.](image)

Conclusions:
Insights into the interaction of human estrogen receptor, hERα, with its benzophenone derivatives the morpholine ether and p-benzoyl-L-phenylalanine BPs, (1-4)b,c, were elucidated through molecular docking and molecular dynamics study. Docking study using 17β-estradiol as ligand was able to reproduce the binding orientation observed in the crystal structure of hERα obtained from the Protein Data Bank with the ID code 1A52, thus supporting the validity of our docking protocol. The docking studies of benzophenone derivatives revealed that polar (Arg394, Glu353, His524, Asp351 and Lys529), aromatic (Phe404, Trp383) and non-polar (Ala350, Met421, Leu346, Leu354, Leu384, Leu387, Leu391, Leu394, Leu428, Leu525, Ile424) amino acid residues play important roles in the stabilization of hERα-BPs complex. The structure-modification relationship of these BP molecules can be used in the developing new and potential breast cancer drug. The results are indicating that hERα-4c exhibited lowest binding energy, \( \Delta G = -11.35 \text{ kcal/mol} \) compared to p-benzoyl-L-phenylalanine BPs (1-4)b and the value is comparatively lower compare to the natural estrogen, 17β-estradiol, \( \Delta G = -9.67 \text{ kcal/mol} \). Docking experiments for the BPs with the hERα binding pocket highlighted the necessity of having a hydroxyl group to permit proper interaction between BPs and hERα while the addition of alanine group on ring A is essential for maximum estrogenic activity and this further lower the binding energy of the hERα-BP complex. To further understand the structural effects of the ligand binding, we performed a 10 ns molecular dynamic simulation study on 4c bound to hERα dimer using AMBER FF99SB force field available in Gromacs 5.0.7 program. The results confirms the previous docking results that the amino acid residues of hERα participated in hydrogen bonding formation with the ligand. The flexibility of Helix 12 (H12) in the 4c bound hERα dimer which is responsible for the structural change is in keeping with the large change in the root mean square deviations (RMSDs), the average of 0.18-0.2 nm and the high value of radius of gyration, Rg, 2.35 ± 0.01 nm. The simulation results also reveal that H12 in chain B, which is the hERα monomer with the ligand, is stable while H12 in chain A i.e. the hERα monomer without the ligand, is more dynamic. However, no sign of conformational change towards an agonist or antagonist structure was detected probably due to short simulation time. In order to elucidate the mechanism of inhibition by 4c, longer simulation time is required. We are also considering two 4c ligands with dimer hERα and currently the MD simulation with longer simulation time is being carried out. The structure-activity relationship of the designed BP should be helpful for the search of a new potent BP derivative and this could lead to the discovery of a better human estrogen receptor (the human ERα inhibitor) with different modes of action. In addition to that, the future works to be conducted include the synthesis of the hydroxyl derivative of benzophenone morpholine ether, 4c. Investigation on its
anticancer property and its ability to suppress cancer cell in vitro will be conducted and compared against the natural estrogen and other anticancer drugs.

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