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Designer interface peptide grafts target estrogen receptor alpha dimerization

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Abstract

The nuclear transcription factor estrogen receptor alpha (ER α), triggered by its cognate ligand estrogen, regulates a variety of cellular signaling events. ER α is expressed in 70% of breast cancers and is a widely validated target for anti-breast cancer drug discovery. Administration of anti-estrogen to block estrogen receptor activation is still a viable anti-breast cancer treatment option but anti-estrogen resistance has been a significant bottle-neck. Dimerization of estrogen receptor is required for ER activation. Blocking ER α dimerization is therefore a complementary and alternative strategy to combat anti-estrogen resistance. Dimer interface peptide “I-box” derived from ER residues 503–518 specifically blocks ER dimerization. Recently using a comprehensive molecular simulation we studied the interaction dynamics of ER α LBDs in a homo-dimer. Based on this study, we identified three interface recognition peptide motifs LDKITDT (ER α residues 479–485), LQQQHQLAQ (residues 497–506), and LSHIRHMSNK (residues 511–520) and reported the suitability of using LQQQHQLAQ (ER 497–506) as a template to design inhibitors of ER α dimerization. Stability and self-aggregation of peptide based therapeutics poses a significant bottle-neck to proceed further. In this study utilizing peptide grafted to preserve their pharmacophoric recognition motif and assessed their stability and potential to block ER α mediated activity *in silico* and *in vitro*. The Grafted peptides blocked ER α mediated cell proliferation and viability of breast cancer cells but did not alter their apoptotic fate. We believe the structural clues identified in this study can be used to identify novel peptidomimetics and small molecules that specifically target ER dimer interface generating a new breed of anti-cancer agents.

Keywords

Estrogen receptor; Antiestrogen resistance; Interface peptides; Breast cancer; Cell proliferation

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Conflicts of interest
None.

1. Introduction

Estrogen receptor alpha (ER α) is a ligand-triggered nuclear transcription factor that regulates the expression of various target genes as well as a wide range of cellular signaling events, such as growth, development, and survival [1]. ER α is a modular protein containing five major domains: an N-terminal activation function-1 domain (AF-1), a DNA-binding domain (DBD), a hinge region, a C-terminal ligand-binding domain (LBD) and an activation function-2 domain (AF2) [2,3]. Estrogens bind to the LBD of ER α and trigger a cascade of conformational changes which results in receptor dimerization, translocation to the nucleus, and binding to the estrogen response element (ERE) of the target genes resulting in the activation of gene transcription [4–6]. ER α is expressed in about 70% of breast tumors and their growth and survival are driven by estrogens [1,7]. Anti-estrogen tamoxifen has been administered as an effective treatment for ER α -positive breast tumors of both pre-menopausal stage and postmenopausal stage [8]. However, many ER α positive breast tumors are intrinsically resistant to tamoxifen, despite high expression of ER α . Breast tumors that are not intrinsically resistant to tamoxifen will eventually acquire resistance in advanced stage of tumor progression [9,10]. The mechanisms of resistance have not been clearly understood, hormone independent activation and protein-protein interactions with coregulator proteins of ER α may play a role in anti-estrogen resistance [11]. All hormone dependent drug discovery strategies relying on ligand competition at the estrogen binding site will face similar issues highlighting the importance of identifying new hormone independent strategies. Receptor dimerization is a required step for transactivation and therefore inhibiting dimerization using peptides derived from dimer interfaces is an attractive alternate to identify ligand-independent inhibitors. Yudit and Koide [12] blocked ER α transactivation using I-box peptide which has a strong homology with sequence 503–518 of ER α . The I-box peptide is very specific to ER α and does not alter the activities of other nuclear receptors but highly susceptible to self-aggregation [12]. Recently analyzing the dimer interface contacts between two ER α LBDs in a homo-dimer we identified three interface recognition peptides LDKITDT (ER α residues 479–485), LQQQHQLAQ (residues 497–506), and LSHIRHMSNK (residues 511–520) and reported the suitability of using LQQQHQLAQ as template to design inhibitors of ER α dimerization [13]. In this study, utilizing peptide grafting techniques and structure-based optimization of the synthetic dimer interface peptides to preserve their pharmacophoric interaction motif, we demonstrate their ability to block ER α mediated activity *in silico* and *in vitro*.

2. Materials and methods

2.1. Reagents

Estradiol-17 β and 4-hydroxy tamoxifen were obtained from Sigma. The peptides used in these studies were made by Anaspec Inc. (Fremont, CA). Cell culture media and reagents were purchased from Life Technologies.

2.2. Modeling the grafted peptide

The key residues from “LQQQHQLAQ” interface recognition motif was grafted within a poly-ALA or poly-GLN helix while preserving the pharmacophoric ER dimer recognition

motif to generate four designer α -helical peptides using PyMol modeling interface [14]. Knowledge based grafting was carried out based on observing the ER dimer interface and the recognition motif sequence to alter residues to enhance interactions. These are PI: AAAHQALAQAAAAAAAAA; PII: AAADQADAQAAAAAAAAA; PIII: QQQQQQQHQQLAQQQQQQQ and PIV: QHQQQQQDQQLAQQQQQQQ. Energy minimization and dynamics of all the four grafted peptides were studied using molecular dynamics simulation using GROMACS package [15,16] with OPLS force field [17]. Initially, the four grafted peptides were energy minimized *in vacuo* followed by another short energy minimization in the presence of SPC water using steepest descent algorithm. The solvated system was equilibrated at 300 K with a short 100 ps position-restrained dynamics which is followed by a short NVT and NPT simulation. Final production level simulations were performed in the isothermal isobaric (NPT) ensemble at 300 K, using an external bath with a coupling constant of 0.1 ps. Pressure was kept constant (1 bar) by using the time constant for pressure coupling was set to 1 ps. The LINCS algorithm was used to constrain the bond lengths involving hydrogen atoms, allowing the use of a 2 fs time step.

2.3. Modeling the ER α -peptide complexes

The test peptides were manually docked to the ER α dimerization interface. The resulting protein-peptide complexes were then subjected to energy minimization followed by molecular dynamics simulation as described in 2.2. Analysis of the trajectory and energetics were performed using GROMACS analysis tools. Secondary structure calculations were carried out by the DSSP [18] module integrated within GROMACS. Visualization of the structures was carried out using PyMol [14] and VMD [19].

2.4. Cell culture

MCF-7 and MDA-MB-231 were originally obtained from the American Type Culture Collection ATCC. Cells were cultured according to the suggested ATCC protocol as follows: 4×10^4 cells were seeded in T-75 vented flask and grown to 70–80% confluency in Minimum Essential Medium (MEM, Life Technologies) supplemented with 10% fetal bovine serum (Corning), 0.01 mg/ml of human recombinant insulin (Life Technologies) and 5% penicillin streptomycin (Corning). Cells were housed at 37 °C with 5% CO₂ and 10% humidity. Two days prior to the treatments cells were changed to phenol-free IMEM media.

2.5. Cell viability

Cell viability was assessed using a conventional MTT assay. A seed density of 80×10^4 was added to each well of a clear 96 well plate (Corning) in phenol-free IMEM supplemented medium. The cells were incubated overnight at 37 °C with 5% CO₂ and 10% humidity. The cells were treated with 0.1, 1 or 5 μ M of test peptide and/or 1 nM E2. Control wells were treated with 0.5% DMSO. Each experimental condition was done in triplicates. The cells were treated for 24 h at 37 °C with 5% CO₂ and 10% humidity. Thiazolyl bromide (1 mM) was added to each well, excluding negative control wells, to which IMEM supplemented medium was added. The cells were incubated with thiazolyl bromide for 4 h. The media was removed and replaced with 0.1 N HCl in 2-propanol and the absorbance at 570 nm and 650 nm was monitored using the Bio-Tek Synergy microplate reader.

2.6. Cell proliferation

Cell proliferation was assessed using a fluorescent ethynyldeoxyuridine (EdU) incorporation assay. A seed density of 80×10^2 cells in phenol-free IMEM supplemented media was added to each well of a 96 well black side, clear bottom plate (Corning). The cells were incubated overnight at 37 °C with 5% CO₂ and 10% humidity. The cells were treated with 1 μM of test peptide and/or 1 nM E2. Control wells were treated with 0.1% DMSO. Each experimental condition was done in triplicates. The cells were treated for 8 h at 37 °C with 5% CO₂ and 10% humidity. 10 μM EdU (5-ethynyl-2'-deoxyuridine, Life Technologies) was added to each well, except for negative control wells in which IMEM medium was added instead. The cells were incubated with EdU for 1 h at 37 °C with 5% CO₂ and 10% humidity. The medium was then removed, and the cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature and washed with PBS twice. Next the cells were permeabilized with 0.1% Triton-X 100 in PBS for 15 min at room temperature and washed twice with PBS. EdU incorporation was detected employing a click-it reaction. The click-it reaction mixture was prepared according to the protocol suggested by Life Technologies and the cocktail contained CuSO₄ (Life Technologies) and Alexa Fluor 488 azide (5-Carboxamido-(6-Azidohexanyl), Bis(Triethylammonium Salt)), 5-isomer (Life Technologies). The click-it cocktail was then incubated with cells for 30 min at room temperature in the dark. After the Click-iT cocktail was removed the cells were washed Click-iT reaction rinse buffer (Life Technologies). HCS NuclearMask blue stain (diluted 1:2000 in PBS, Life Technologies) was then incubated with the cells for 30 min at room temperature in the dark. The cells were washed twice with PBS. The fluorescence of each well was then quantified with the Bio-Tek Synergy microplate reader employing the 495 nm excitation and 519 emission filters and representative images of each well were captured with the Zeiss Axio Observer Inverted Microscope. Images were analyzed using Image J software.

2.7. Flow cytometry

Cells with seed density of 5×10^5 will be added to T-25 vented flask in phenol-free IMEM supplemented medium and allowed to grow for 70–80% confluency. The cells were then treated with 1 μM test peptide and/or 2 nM E2 for 16 h or 24 h and incubated at 37 °C with 5% CO₂ and 10% humidity. The floating cells from each condition were stored in conical tubes and the attached cells were washed with PBS (Corning) and removed by incubating them with 0.25% trypsin 2.21 mM EDTA solution (Corning). Trypsinized and removed from the cells by centrifuging for 5 min at 1000 RPM and aspirating the supernatant. The cells were resuspended in binding buffer (10 mM HEPES/NaOH pH7.5 containing 0.14 M NaCl and 2.5 mM CaCl₂) and transferred to test tubes. Annexin V FITC Conjugate (1 μg/ml) (Sigma) and Propidium Iodide (1 μg/ml) (Sigma) were added to all tubes, including an untreated control, except in the untreated, unstained control. The cells were incubated with the dyes for 10 min in the dark. Flow cytometry was performed using a Becton Dickinson Fortessa SORP 14-color flow cytometer equipped with a 488 nm excitation filter, a 515/20 nm emission filter and a 610/20 nm emission filter.

3. Result and discussion

3.1. Generating dimer interface peptide grafts

Short peptide sequences derived from protein-protein interfaces often do not retain the structural motif required and could result in the loss of favorable binding enthalpy [20]. Based on the stability and interaction profiles obtained from the molecular dynamics (MD) simulations, we have identified that ER α residues 497–506 (LQQQHQLAQ) could bind at the ER α dimer interface and potentially block ER dimerization [13]. However, this short peptide sequence does not present the core recognition motif in its native conformation [13]. To provide additional structural stability and improve interaction features, we have grafted four peptides PI-IV as follows PI: AAAHQALAAAAA; PII: AAADQADAAAAA; PIII: QQQQQQHQLAQ and PIV: QHQQQQDQLAQ. These peptides present the core pharmacophoric motif as in the original protein and can potentially bind to the ER α dimer interface groove formed by helices 9 and 10/11 with enhanced affinity and prevent ER α dimerization. In peptide I, the core recognition motif is grafted within an alanine (ALA)- rich sequence to render a Poly-ALA helix with enhanced helical propensity. Three N-terminal ALA and the C-terminal ALA-tail have been added to provide structural stability to generate an 18-mer peptide. Peptide II was generated by replacing HIS 501 and LEU 504 of the core motif in peptide I with aspartic acid. This alteration not only enhances the polarity of the peptide but also generates a region of negative electrostatic potential surface that is complementary to the groove created by basic residues of Helix 10 at the interface. ER dimer interface peptide motif residues contain many glutamine residues [13]. To mimic this feature, a couple of peptides were grafted with a glutamine (GLN) rich or poly-GLN template. Peptide III, a 19-mer was generated by burying the core recognition motif within a Poly-GLN helix. Peptide IV was generated by replacing histidine of peptide III with an aspartic acid such that it can interact with the region of positive electrostatic potential contributed by the basic residues of Helix 10 at the interface.

3.2. Structural stability and conformational dynamics of ER α inhibitor peptides

To act as a peptidic inhibitor of ER α , the peptide must be stable in helical form such that it retains its pharmacophoric feature necessary to bind to the appropriate location at the dimer interface [12,13]. To analyze the structural stability of all the four designed peptides, the grafted peptides were modeled as helical peptides and their structural stability were studied by performing 5 ns molecular dynamics simulation in explicit water. The simulations done in explicit water conditions are computationally intensive and presents a realistic solvent environment to peptides and peptide-protein complexes compared to gas phase calculations. The MD average structure of Peptide I reveals that the C-terminal ALA-tail loses its helicity during the simulation but its N-terminal region is strongly helical and particularly the core recognition region “HQALAQ” colored in purple is helical (Fig. 1AI). Secondary structure analysis reveals that the loss of helicity in the C-terminal region is mostly prominent during the last nanosecond of the simulation while the N-terminal region including the core recognition region is predominantly α -helical throughout the simulation (Fig. 1AII). Significant reduction in the helicity has been observed for peptide II post 3 ns simulations, with its N-terminal region fluctuating between a 3_{10} -helix or helical turn conformation,

essentially rendering a partially helical peptide (Fig. 1BI, II). Helicity of peptide III was conserved throughout the simulation period (Fig. 1CI, II). On the other hand, peptide IV could maintain its helical topology only up to 1.5 ns and recovers to a kinked helical structure during the last ns of the simulation (Fig. 1DI, II). This kink separates the N-terminal helix from C-terminal helix thereby significantly perturbing the ER α dimer interface recognition motif.

3.3. Structural stability of ER α -inhibitor peptide complexes

We hypothesized that maintaining the helical topology alone is not a sufficient criterion for the interface peptide to act as an inhibitor; it must form a stable complex with ER α and maintain its pharmacophoric feature necessary for ER α recognition throughout the simulation. To test this hypothesis, each of the four grafted peptides was docked at the dimer interface of ER α monomer and then the stability of each protein-peptide complex was analyzed post 10 ns all-atom molecular dynamics simulations. Fig. 2 represents variations of two structural parameters, root mean square deviation (RMSD) and radius of gyration (Rg), of all the four ER α -peptide complexes (A and C) and peptides (B and D) when bound at the ER α dimer interface. The RMSD of the backbone C α atoms of the simulated system over time can be used to analyze the structural stability of the system while the 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. During the first 2 ns of the simulation, all the four ER α -peptide complexes undergo conformational readjustments to its environment prior to reaching equilibria (Fig. 2A). RMSD profile indicates formation of stable ER-peptide complexes, except for peptide III. Peptide I and II, in particular, form highly stable complexes with ER α as evident from the stable Rg profile throughout the simulation (Fig. 2C). After attaining the equilibrium state the ER α -peptide III complex remains stable until 7 ns, beyond which it undergoes significant structural changes marked by a rapid increase in RMSD (Fig. 2A). Radius of gyration of the ER α -peptide III complex increases continuously throughout the simulation and never stabilize during the simulation (Fig. 2C). Interestingly, peptide IV retained its initial kinked helical conformation throughout the simulation. Fig. 2B and D represents the structural changes of the inhibitor peptides compared to their initial seed conformation as adjudged by the RMSD and Rg profiles. Peptides I and IV showed significant retention of their seed conformation. As expected Peptide III, showed higher fluctuations and never stabilized during simulation, indicating significant conformational changes during the simulation time scale. The profiles for peptide II indicate that it underwent significant conformational changes post binding (Fig. 2B and D).

A closer look at the secondary structure profiles of the peptides during simulation indicates that only peptide I maintains a predominantly helical structure throughout the simulation (Supplementary Fig. 1A–D). Peptides II and III remain helical only during initial 2 ns of the simulation and keeps fluctuating between helical turn or 3_{10} / π -helix with a brief recurrence of α -helical structure during the rest of the simulation period. Peptide IV lost its seed secondary structure during the equilibration period and exhibits bend/turn propensity throughout the simulation period (Supplementary Fig. 1D). The comparatively stable bend

like structure justifies the relatively stable observed RMSD and Rg profile during simulation (Fig. 2).

3.4. Analysis of ER α -peptide interactions

Average interaction energy components (coulomb and van der Waals interaction energy computed by Lenard-Jones potential) between each of the bound peptide and ER α have been calculated during the last 8 ns of the simulation (conformational equilibration of the trajectories) and summarized in Table 1. Peptide I-III bound to the ER α dimer interface with comparable LJ interaction energies. Replacement of histidine and leucine corresponding to ER residues HIS501 and LEU504 with aspartic acid in peptide II enhances the interaction energy significantly, particularly its coulomb interaction energy contribution. Peptide IV which lost its secondary structure during simulations renders interactions that were non-specific. The pharmacophoric feature for its ER α dimer interface recognition is not maintained throughout the simulation. Fig. 3 illustrates potential interactions of Peptide I at the core dimerization groove formed by helix 9 and helix 10/11. ILE487, LEU497 from helix 9 and three leucine residues (LEU504, LEU508, LEU511) from helix 10/11 tightly interacts with the bound peptide (Fig. 3A). In addition to these hydrophobic interactions, peptide I also participates in hydrogen bonding interactions with ER residues GLN498 and GLN500 from helix 10 (Fig. 3B).

3.5. Effect of peptides in regulating estrogen (E2)- ER α mediated activities in vitro

In order to determine if the grafted peptides I and II alter breast cancer cell viability, MCF7 (ER positive) and MDA-MB-231 (ER negative) breast cancer cells were subjected to the test peptides for 24 h in the presence and absence of 1 nM E2. The MTT assay was used to determine the number of viable cells. Peptides I and II did significantly reduce the number of viable MCF7 cells in the presence of E2. The peptides did not significantly affect MDA-MB-231 breast cancer cell viability at the 5 μ M concentration (Supplementary Fig. 2). This indicates the peptides' selectivity towards estrogen receptor expressing MCF-7 cells.

The immediate next step was to determine if these peptides alter estrogen receptor mediated breast cancer cell proliferation. MCF7 breast cancer cells were subjected to 1 μ M treatments of the peptides in the presence or absence of E2 (1 nM) or 4OHT (1 μ M). After 8 h the effect of treatments were identified using fluorescence microscopy and a 5-ethynyl-2'-deoxyuridine (EdU) Click-iT HCS cell proliferation assay. Image analysis of captured images from EdU incorporated MCF-7 breast cancer cells showed a significant decrease in estrogen induced cell proliferation (4A & B). Fluorescent high-throughput imaging (HCS) assay showed that the peptides I and II significantly inhibited E2 (1 nM) induced MCF7 cell proliferation (Fig. 4C). In addition, these peptides had no effect on MCF7 breast cancer cell proliferation in the absence of any other treatments.

While the dimer interface peptides attenuated E2 induced MCF7 breast cancer cell proliferation and cell viability, these peptides did not induce apoptosis of breast cancer cells. MCF7 and MDA-MB-231 (negative control) breast cancer cells were subjected to 1 μ M test peptide (I or II) in the presence of 2 nM E2 treatments for 16 h and the number of cells undergoing apoptosis was evaluated with annexin V and propidium iodide staining using

flow cytometry. None of the test compounds induced apoptosis in either MCF7 or MDA-MB-231 cells (Supplementary Fig. 3).

4. Conclusion

Based on our *in silico* and in vitro observations, it is evident that the grafted peptides derived from ER dimer interface could potentially block homo-dimerization of the receptor a crucial step in mediating transactivation of target genes. Grafted peptides (I and II) blocked ER mediated cell viability and proliferation but did not alter apoptosis, indicating that these designer peptides will probably be cytostatic as opposed to cytotoxic agents. We believe the structural clues identified in this study can be used to identify novel peptidomimetics and small molecules that specifically target ER dimer interface generating a new breed of anti-cancer agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2016.07.083>.

Fig. 1.

Left panel: MD average structure of the four peptide in solution; Right panel: Evolution of secondary structure during simulation. A: Peptide I; B: Peptide II; C: Peptide III; D: Peptide IV.

Fig. 2. Variations of the RMSD and Rg during simulation time. A & C represents the RMSD and Rg profile all the four ERα-peptide complexes, respectively. B & D represents the RMSD and Rg profile all the four peptides when bound with ERα.

Fig. 3. Details of the van der Waals (A) and hydrogen bonding interactions (B) of the peptide I with ERα obtained from 10 ns MD simulation. Peptide I is colored blue while ERα is colored green. Interacting van der Waals residues are rendered as surface and colored red. Residues involved in the hydrogen bonding interactions are shown in stick representation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Effect of ER interface peptides on MCF-7 breast cancer cell proliferation. A. Representative images from the Click-iT EdU-AlexaFluor 488 incorporation assay when MCF-7 breast cancer cells were treated with 1nM estradiol with or without ER interface peptide PI. The images were captured with a Zeiss Axio Observer inverted microscope with filters appropriate for Alexa Fluor 488- ex495nm, em519nm and NuclearMask (H10325) Blue stain- ex350nm, em461nm. The total DNA content was stained with a NuclearMask Blue stain (blue), and EdU was visualized after conjugation with Alexa Fluor 488 azide (green). Peptide treatment significantly reduced EdU incorporation (B). C. ER interface peptides (PI and PII) decrease estrogen induced cell proliferation of MCF-7 breast cancer cells in a High-throughput (HCS) Click-iT imaging assay. Fluorescence intensity was captured employing 497 nm excitation and 519 emission filters with the BioTek Synergy plate reader. Error analysis was performed using Graphpad Prism. Results represent mean $\pm$ standard deviation, where ** or †† represent $p < 0.01$. (* was use to identify statistical significance of treatment vs vehicle comparison and † comparisons between estrogen treatments). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Comparison of average Coulomb, Van der Waals, and total interaction energies in units of KJ/mol for all the four peptides with ER α LBD, obtained from the MD simulation.

Protein-peptide complexes	Average coulomb interaction energy (kJ/mol)	Average LJ interaction energy (kJ/mol)	Total interaction energy (kJ/mol)
ER α -Peptide I	-131.41	-194.02	-325.43
ER α -Peptide II	-332.28	-199.94	-532.22
ER α -Peptide III	-145.87	-171.26	-317.13
ER α -Peptide IV	ns	ns	ns

*
ns - non-specific interactions.