Bioorganic & Medicinal Chemistry Letters 22 (2012) 1743–1746

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of a spin-labeled anti-estrogen as a dynamic motion probe for the estrogen receptor ligand binding domain

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ARTICLE INFO

Article history:
Received 18 November 2011
Revised 14 December 2011
Accepted 19 December 2011
Available online 8 January 2012

Keywords:
Nitroxide spin label
Estrogen receptor-ligand binding domain
Selective estrogen receptor modulator
Anti-estrogen
Dynamic motion probe

ABSTRACT

The preparation and characterization of a novel nitroxide spin probe based on a steroidal anti-estrogen is described. The probe **5** demonstrated very high binding affinity for both the alpha and beta isoforms of the estrogen receptor–ligand binding domain. EPR spectrometric studies demonstrate conformational constraints for the ligand, consistent with the nitroxyl moiety occupying a position just beyond the receptor-solvent interface.

Estrogen receptor-alpha (ERα) is a member of the family of nuclear hormone dependent transcription factors responsible for mediating the effect of endogenous estrogens.[1] Because of its important role in hormone responsive breast cancer,[2,3] significant efforts have been directed toward elucidating conformational changes elicited by complexes between this protein and its natural and synthetic ligands. The folding of helix 12, located at the C-terminus of ERα–ligand binding domain (ERα–LBD), after ligand binding, is a critical factor in determining an agonist or antagonist response[4–6] through recruitment of either coactivators or corepressors, respectively. In the agonist conformation, helix 12 folds into a compact structure, and the 'LxxLL' binding motif of activation function 2 (AF2) is exposed on the surface where coactivator proteins bind, promoting transcriptional activity, including cell proliferation. However, in the antagonist conformation, the folding of helix 12 is disrupted, causing AF2 to be buried within the receptor, thereby preventing coactivator recruitment and inhibiting cell proliferation. This is the mechanism by which selective estrogen receptor modulators (SERMs), such as Tamoxifen or Raloxifene, exhibit their beneficial pharmacological effects.

X-ray crystallography[4,7] and nuclear magnetic resonance spectroscopy[8] (NMR) are the primary techniques from which most conformational data regarding ligand–ERα–LBD complexes have been drawn. However, crystallography requires crystallization of the complex under nonphysiological conditions and more importantly, only provides a conformational 'snapshot' of the complex, providing little or no information about the dynamic nature of the interactions. These interactions can be simulated using molecular modeling,[9] thereby providing an approximation of the process and surrounding environment. NMR spectroscopy complements in silico studies by providing additional data reflecting the dynamics of the ligand–receptor complex, but is limited by the size of the biomolecule.

Electron paramagnetic resonance (EPR) spectroscopy is a sensitive technology for studying protein dynamics. EPR typically employs nitroxide radicals as the paramagnetic species, which, due to their biological stability, makes them ideal for studying ligand–receptor interactions at a molecular level under physiological conditions. Nitroxide radicals also possess T_1 contrast properties extending their application into magnetic resonance imaging (MRI).[10] This particular property allows for the potential use of spin labeled ligands in vitro and in vivo molecular imaging studies.

To date most research on ERα–LBD, and other receptors, using EPR has used site directed spin labeling (SDSL) where the nitroxide label is attached to a specific amino acid on the receptor,[11] rather than using a labeled ligand. When both ERα–LBD and ligand are labeled, interspin distances can be determined through double

Abbreviations: ERα, estrogen receptor alpha; ERα-LBD, estrogen receptor alpha-ligand binding domain; AF2, activation function 2; SERM, selective estrogen receptor modulator; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; SDSL, site directed spin labeling; DEER, double electron-electron resonance; RBA, relative binding affinity; ERβ, estrogen receptor beta.

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doi:10.1016/j.bmcl.2011.12.091

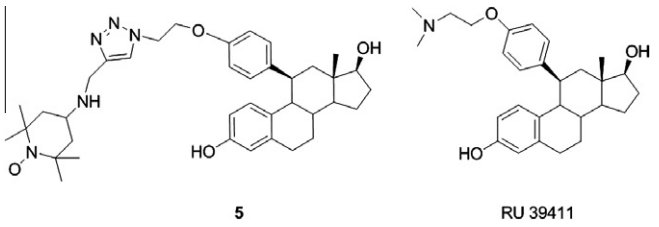
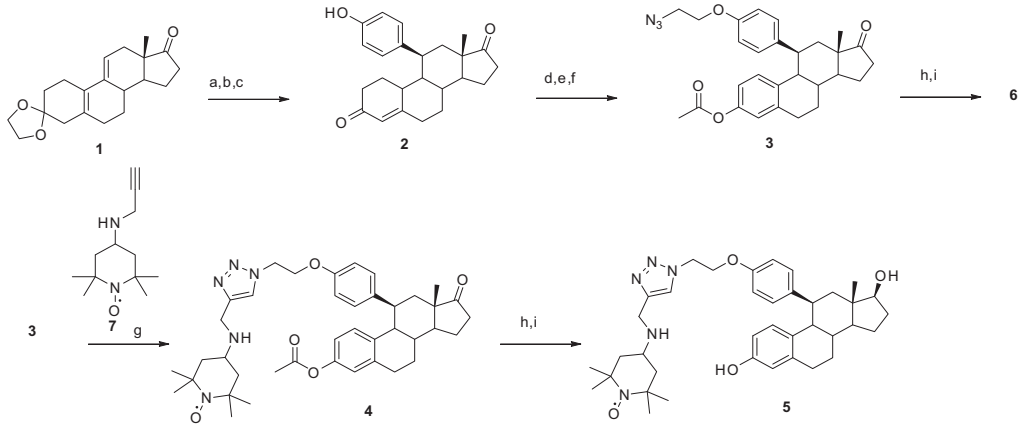


Figure 1. Target compound **5** designed from RU 39411.

electron–electron resonance (DEER) spectroscopy. This approach provides not only spatial information but also insight regarding the biological microenvironment, such as polarity and proticity.[12] Previous work has demonstrated that 11β-functionalized estradiols generate ERα antagonists[13,14] while maintaining high affinity for the receptor.[15] Judicious modifications of the synthesis would permit facile entry into anti-estrogens bearing the requisite spin label. In this study, we describe the preparation and characterization of a molecular EPR probe for ERα designed to address this 'gray area' left by modeling, NMR and crystallography.

The target for our study, compound **5**, shown in Figure 1, combines the high affinity antiestrogenic component associated with RU 39411[16] and the TEMPO spin label, linked together via a triazole. The spin label **7** was prepared in 91% yield via reductive amination of commercially available 4-oxo-TEMPO with propargyl amine in the presence of acetic acid in 1,2-dichloroethane.[16] The complementary azido-estradiol derivative was synthesized, as outlined in Scheme 1. Starting from estradiene dione-3-ketal, **1**, regioselective epoxidation[17] of the 5,10 olefin using hexafluoroacetone and 50% hydrogen peroxide afforded a 3:1 mixture of α:β isomers in 56% overall yield. Separation of the isomers was critical as only the α isomer gave the desired 11β intermediate. The Grignard reagent of 4-bromo(phenoxy) trimethylsilane was prepared and used immediately undergoing a copper (I) mediated 1,4-addition to the pure α-epoxide which, after acid hydrolysis, afforded 11β-(4-hydroxyphenyl)estra-4,9-diene-3,17-dione **2** in 87% yield.[13] 11β-(4-hydroxyphenyl)estra-4,9-diene-3,17-dione underwent alkylation with ethylene glycol ditosylate,[18] followed by the displacement of the terminal tosylate by sodium azide. Aromatization of the A ring using acetic anhydride and acetyl bromide in dichloromethane furnished 11β-(2-azidoethoxyphenyl-3-acetoxyl-estra-1,3,5(10)-trien-17-one **3**. Huisgen 3+2 cyclization of **3** with 4-propargylamino-2,2,6,6-piperidine-N-oxide **7** followed by reduction and saponification gave the spin labeled antiestrogen **5** in a 71% yield (3 steps). Overall yield for 11 steps was 12%. Reduction and saponification of **3** afforded 11β-(4-azidoethoxy)phenyl estradiol **6** as the reference anti-estrogen.



Scheme 1. Synthesis of spin labeled 11β-estradiol derivative **5** and parent steroid **6**. Reagents and conditions: (a) H_2O_2 (50%, 2.3 equiv), $CF_3C(O)CF_3$ (0.11 equiv), pyridine(0.09 equiv), DCM, 0→23 °C, 12 h; (b) (i) Mg (1.7 equiv), I_2 (catalytic), 4-bromo(phenoxy) trimethylsilane (1 equiv), THF, 65 °C, 5 h (ii) Cu(I)Cl (0.1 equiv), THF, −10 °C→23 °C, 12 h; (c) 70% AcOH 30% H_2O, 60 °C, 1.5 h; (d) ethylene glycol ditosylate (1.5 equiv), K_2CO_3 (4.0 equiv), 80 °C, 12 h.; (e) NaN_3 (4.0 equiv), EtOH, 80 °C, 6 h; (f) Ac_2O (1.0 equiv), AcBr (2.5 equiv), DCM, 23 °C, 6 h.; (g) Cu_2SO_4 (0.01 equiv), $NaC_6H_7O_6$ (0.05 equiv), 1:1 *t*-butanol–water, 12 h; (h) $NaBH_4$ (1.2 equiv), MeOH (i) NaOH (4.0 equiv), MeOH.

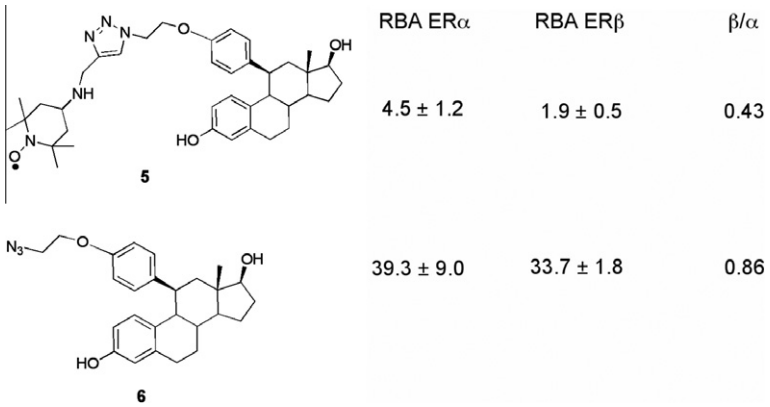


Figure 2. Relative binding affinities (RBA) of spin probe **5** and its parent steroid **6**.

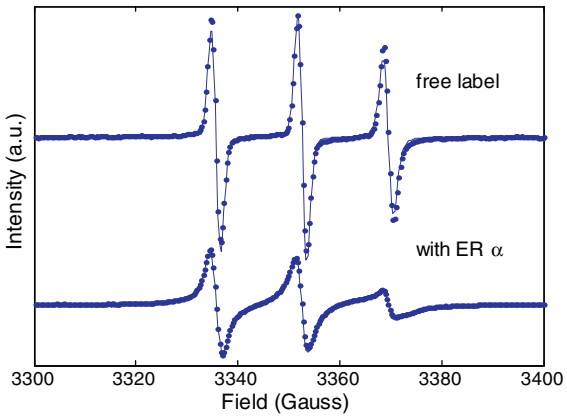


Figure 3. EPR spectra of free **5** (top) and **5** in the presence of ERα–LBD (bottom). Lines show least-squares fits to the experimental data as detailed in the Supplementary data.

as $N = R_{||}/R_{\perp}$ from 1 (isotropic motion) for the free label to 16 in the presence of ERα–LBD, with the axis of fastest rotation lying along the N–O bond of the nitroxide. The complete set of parameters obtained from the least-squares lineshape fitting of the data is given in the Supplementary data.

The EPR results are consistent with the binding mode predicted for the label **5** based on molecular docking studies, as shown in Figure 4. The steroidal(estradiol) moiety of the spin-labeled compound (shown in yellow) occupies the same position as tamoxifen bound in the antagonist conformation of ERα–LBD (shown in green). One should note the position of the triazole ring which occupies the same region as the basic dimethylamino group of tamoxifen. This binding mode places the triazole at the receptor-solvent interface, with the TEMPO group extended beyond the binding pocket, allowing relatively rapid motion of the nitroxide around the axis parallel to the N–O bond.

In summary, we have synthesized, characterized and evaluated a high affinity spin labeled anti-estrogenic ligand designed to study

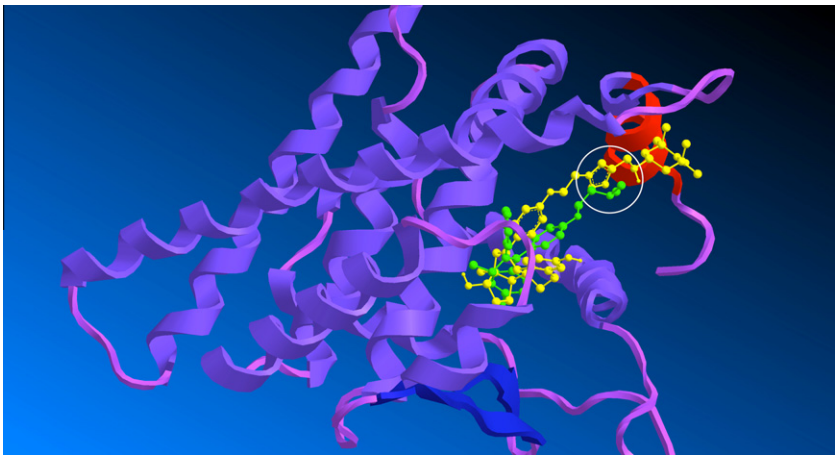


Figure 4. Binding poses of tamoxifen (green) and **5** (yellow) after molecular docking with ERα–LBD monomer, illustrating the disruption of Helix 12 (red).

Relative binding affinities (RBA) of spin probe **5** and azido-anti-estrogen **6** as ligands for ERα–LBD and ERβ–LBD were determined using a competitive binding assay, the results of which are given in Figure 2. The RBA values for the azidoethoxy derivative **6** (39% and 34%) are comparable to those reported for RU39411,[15,19] the dimethylaminoethoxy analog. Incorporation of the spin label reduces affinity of **5** by less than an order of magnitude (RBA = 4.5%) toward ERα–LBD, indicating that extension of the substituent does not completely abolish binding within the ligand binding pocket. The affinity at the ERβ–LBD was lower (RBA = 1.9%) indicating that binding of the larger substituent is somewhat less favored at this subtype. While the terminal azido group was well tolerated, introduction of the substituted triazole moiety did affect the affinity somewhat, but overall antagonist activity was maintained. Based on these results, we proceeded to evaluate **5** as a spin labeled molecular probe for ERα–LBD.

An initial characterization of the binding of **5** to the ERα–LBD was made by standard continuous wave EPR at 9.5 GHz. Figure 3 compares spectra of free **5** in solution (top) and **5** in the presence of ERα–LBD (bottom). Although both spectra exhibit the three lines characteristic of fast probe motion, the major component exhibits lines that are considerably broadened and have different relative peak heights when ERα is present. These changes reflect a decrease in the average rate of rotation by nearly an order of magnitude, from $8.9 \times 10^8\ s^{-1}$ for the free label to $1.2 \times 10^8\ s^{-1}$ in the presence of ERα–LBD, and an increase of the rotational anisotropy, defined the real time response of ERα–LBD upon ligand binding. These results provide the basis for the design and preparation of second generation spin probes for the steroid hormone receptors as complements to current spectroscopic methods.

Acknowledgments

This work was supported by Army CDMRP Contract W81XWH06-1-0551 (D.E.B. and R.H.) and NSF-IGERT Award # 0504331 (J.A.H.) We gratefully acknowledge Professor John A. Katzenellenbogen, whose research group carried out the ER binding assays for the spin labeled anti-estrogen and parent steroid.

Supplementary data

Supplementary data (includes detailed experimental procedures, spectra and characterization data, and lineshape fitting results) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.12.091.

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