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In vitro and *in silico* perspectives on estrogenicity of tanshinones from *Salvia miltiorrhiza*

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ABSTRACT

This work aims to investigate the structure-activity relationship for binding and activation of human estrogen receptor α ligand binding domain (hERα-LBD) with tanshinones by a combination of *in vitro* and *in silico* approaches. The recombinant hERα-LBD was expressed in *E. coli* strain. The direct binding interactions of tanshinones with hERα-LBD and their ERα agonistic potency were investigated by fluorescence polarization (FP) and reporter gene assays, respectively. FP assay suggested that the tested tanshinones can bind to hERα-LBD as affinity ligands. Tanshinones acted as agonists of hERα as demonstrated by transactivation of estrogen response element (ERE) in transiently transfected MCF-7 cells and by molecular docking of these compounds into the hydrophobic binding pocket of hERα-LBD. Interestingly, comparison of the calculated binding energies versus Connolly solvent-excluded volume and experimental binding affinities showed a good correlation. This work may provide insight into chemical and pharmacological characterization of novel bioactive compounds from *Salvia miltiorrhiza*.

1. Introduction

Salvia miltiorrhiza, commonly known as Danshen, belonging to the Labiatae family (Chen, Guo, Bao, Lu, & Wang, 2014; Cheng et al., 2012). As one of the most widely used traditional herbs, *Salvia miltiorrhiza* has been officially listed in the Chinese Pharmacopeia (Feng et al., 2011) and applied to clinical practice for more than two thousand years (Liang, Liang, Wang, & Xu, 2009; Tang, Yang, Li, Li, & Chen, 2016). Besides, this medicinal plant is also popularly accepted in many other countries and regions such as the United States and the European Union (Feng et al., 2011). In recent years, more than 100 kinds of constituents have been isolated and identified from *Salvia miltiorrhiza* (Fang, Little, & Xu, 2018). They can be classified into hydrophilic and lipophilic components, generically designated as salvianolic acids (SA) and diterpenoid tanshinones (DT), respectively (Wu, Karioti, Rohr, Bilia, & Efferth, 2016; Xia, Sun, Lou, & Rahman, 2014; Zhou et al., 2018).

Tanshinones are the major lipid-soluble bioactive ingredients of *Salvia miltiorrhiza* (Chang et al., 2014). They are a group of abietane diterpenes including tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone. Structurally, they consist of four rings, such as tetrahydronaphthalene or naphthalene rings A and B, an *para*-quinone or an ortho- or lactone ring C, and a dihydrofuran or furan ring D. Tanshinones have been observed to possess anti-inflammatory, antioxidant and cytotoxic properties to many types of human cancer cells (Bi, Tian, & Row, 2011; Domitrović & Potočnjak, 2016; Don, Shen, Syu, Ding, & Sun, 2006; Li et al., 2014; Lin & Chang, 2000; Zhao, Xiang, Ye, Yuan, & Guo, 2006). Previous studies have demonstrated that they have potential pharmacological efficacy on many diseases, such as inflammation, diabetes, obesity, cancer, tumor, cardiovascular disease, Alzheimer's disease, *etc* (Chen et al., 2017; Cho & Kleeberger, 2015; Liu et al., 2017; Qiu et al., 2017; Wu, Klauck, & Efferth, 2016; Zhang, Hu, et al., 2017.

Based on the structural similarity of tanshinones to endogenous hormones, their biological activities may be induced by binding to nuclear receptors (NRs) (Li, Bonneton, Chen, & Laudet, 2015). As a group of transcription factors, NRs can be activated by their respective functional ligands and then influence the regulation of reproduction, development, homeostasis and metabolism (Dasgupta, Lonard, & O'Malley, 2014). Numerous studies suggested that tanshinones can bind pregnane X receptor (PXR) (Gao et al., 2012), peroxisome proliferator-activated receptor γ (PPARγ) (Guasch et al., 2013), androgen receptor

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(AR) (Xu et al., 2012) and estrogen receptors (ERs) (Li et al., 2015). Estrogens primarily act through two distinct ER subtypes (ERα and ERβ) (Zang, Odwin-Dacosta, & Yager, 2009), which both contain three major functional domains called activation function 1 (AF-1) in the amino-terminal A/B domain, central DNA binding domain (DBD), and carboxyl-terminal ligand binding domain (LBD). The latter two domains can recognize and bind DNA sequences and ligands, respectively (Rastinejad, Ollendorff, & Polikarpov, 2015).

As the main targets of phytoestrogens, ERs have been reported for their ability of binding several ingredients of *Salvia miltiorrhiza* (Li et al., 2015). These bioactive compounds were confirmed to inhibit estrogen-inducible ER transactivation. They might bind and activate estrogen receptors to regulate the transcription of diverse ER target genes, resulting in a wide range of biological effects (Thomas & Gustafsson, 2015). Despite the reported estrogenic effects of several ingredients of *Salvia miltiorrhiza*, the underlying mechanisms of their estrogenicity are still poorly understood.

Hence, in the present work, the binding and activation potency of tanshinones toward human estrogen receptor α (hERα) were investigated by a combination of *in vitro* and *in silico* analysis. Based on the recombinant protein human estrogen receptor α ligand binding domain (hERα-LBD) expressed in *E. coli*, the binding affinities of tanshinones with hERα-LBD were assessed quantitatively by fluorescence polarization assay. Then an estrogen response element-luciferase (ERE-Luc) reporter gene assay was carried out to evaluate the estrogenic activities of tanshinones. In order to reveal the structural basis for their estrogen receptor agonist activities, molecular dynamics simulations were performed to explore the binding modes between tanshinones and the receptor protein.

2. Materials and methods

2.1. Materials

Lipofectamine 2000 transfection reagent, penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Sodium dodecyl sulfate (SDS) was purchased from Amresco (Solon, OH, USA). PageRuler™ Prestained Protein Ladder was purchased from Thermo Fisher Scientific (San Jose, CA, USA). Coumestrol (CS, ≥97.5%), 17β-estradiol (E_2, > 97%), dimethylsulfoxide (DMSO), isopropyl β-D-1-thiogalactopyranoside (IPTG), mouse anti-glutathione S-transferase (GST) monoclonal antibody (mAb) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and TCI (Tokyo, Japan). Tanshinone I (≥98%), tanshinone IIA (≥98%), cryptotanshinone (≥98%) and dihydrotanshinone (≥98%) were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). The structures of tanshinones are shown in Table 1. All other reagents used were of analytical grade.

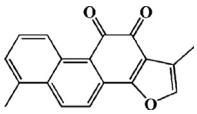
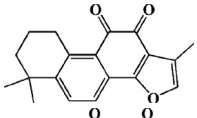
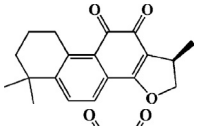
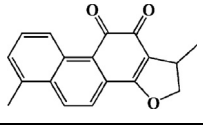
2.2. Preparation of fusion protein

The coding sequence of the N-terminal region of hERα-LBD (residues 282 to 595) was fused with the coding sequence of glutathione S-transferase (GST) and then cloned into the pGEX-4T-1 vector at restriction sites BamHI and XhoI. The expression plasmid pGEX-4T-1-hERα-LBD was introduced into *Escherichia coli* strain BL21(DE3)pLysS. Cells were induced to express hERα-LBD by overnight treatment with 0.5 mM IPTG at 20 °C. The suspension was filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA) to remove all residual bacterial cells. The supernatant was then purified by immobilized metal affinity chromatography (IMAC) on an IDA-Ni^{2+} column (Novagen, Madison, WI, USA). The samples were subjected to 12% SDS-PAGE followed by Western blot analysis.

2.3. Fluorescence polarization competition binding experiment

By employing coumestrol (CS) as a tracer, the binding affinities of tanshinones with hERα-LBD were determined in a competitive fluorescence polarization (FP) assay (Zhang et al., 2018). The protein (250 nM) and the probe (10 nM) were mixed in a total volume of 290 μL and then titrated with various concentrations of tanshinone (10 μL). Each sample was subjected to measuring FP signals after being incubated for 2 h at room temperature. Excitation at 355 nm and emission at 405 nm were monitored by a multi-mode microplate reader (FlexStation 3, Molecular Devices, Sunnyvale, CA, USA). The IC_{50} value (concentration of tanshinone with 50% inhibition of tracer binding occurred) was calculated according to a four parameter logistic equation $Y = (A - D)/[1 + (X/IC_{50})^B] + D$, where Y and X correspond to the polarization value and the tanshinone concentration, A and D are the polarization values at zero and an infinite concentration

Table 1
The molecular length, Connolly solvent-excluded volume (CSEV), IC_{50} values, and docking results for the tested tanshinones.

Compound	Structure	Length (Å)	CSEV (Å^3)	IC_{50} (nM)	Hydrogen bond	Estrogenicity	Binding energy (kcal mol^{-1})
Tanshinone I		10.18	224.6	330.8	—	Agonist	−8.02
Tanshinone IIA		10.08	263.5	120.1	Leu346	Agonist	−8.58
Cryptotanshinone		9.87	271.5	110.3	Leu346	Agonist	−8.79
Dihydrotanshinone		9.63	232.1	125.4	Leu346	Agonist	−8.42