Research Article

Computational and experimental characterization of estrogenic activities of 20(S, R)-protopanaxadiol and 20(S, R)-protopanaxatriol

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Background: As the main metabolites of ginsenosides, 20(S, R)-protopanaxadiol [PPD(S, R)] and 20(S, R)-protopanaxatriol [PPT(S, R)] are the structural basis response to a series of pharmacological effects of their parent components. Although the estrogenicity of several ginsenosides has been confirmed, however, the underlying mechanisms of their estrogenic effects are still largely unclear. In this work, PPD(S, R) and PPT(S, R) were assessed for their ability to bind and activate human estrogen receptor α (hERα) by a combination of in vitro and in silico analysis.

Methods: The recombinant hERα ligand-binding domain (hERα-LBD) was expressed in E. coli strain. The direct binding interactions of ginsenosides with hERα-LBD and their ERα agonistic potency were investigated by fluorescence polarization and reporter gene assays, respectively. Then, molecular dynamics simulations were carried out to simulate the binding modes between ginsenosides and hERα-LBD to reveal the structural basis for their agonist activities toward receptor.

Results: Fluorescence polarization assay revealed that PPD(S, R) and PPT(S, R) could bind to hERα-LBD with moderate affinities. In the dual luciferase reporter assay using transiently transfected MCF-7 cells, PPD(S, R) and PPT(S, R) acted as agonists of hERα. Molecular docking results showed that these ginsenosides adopted an agonist conformation in the flexible hydrophobic ligand-binding pocket. The stereostructure of C-20 hydroxyl group and the presence of C-6 hydroxyl group exerted significant influence on the hydrogen bond network and steric hindrance, respectively.

Conclusion: This work may provide insight into the chemical and pharmacological screening of novel therapeutic agents from ginsenosides.

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1. Introduction

Panax ginseng is a well-known herb in the oriental countries and has been widely consumed in healthy food and as traditional medicine for thousands of years [1]. Previous studies have confirmed its extensive pharmacological effects on the cardiovascular, reproductive, and nervous systems [2,3]. Based on the increasing scientific evidences, it has been used for preventing and treating diseases such as diabetes, cancer, etc [4]. The therapeutic effects of P. ginseng can be attributed to a sort of compounds named ginsenosides, chemically described as ginseng saponins as well. Regarded as the foremost bioactive components in P. ginseng, ginsenosides have been discovered and identified for more than 180 species [5].

Because ginsenosides possess a common four-ring hydrophobic steroid-like structure with sugar moieties attached at C-3, C-6 or C-20 position, they can be divided into four subtypes as protopanaxadiol (PPD), protopanaxatriol (PPT), oleanolic acid, and oleanolic acid [6]. Owing to the diversity of chemical structure, the naturally occurring saponins exhibit a wide range of polarity and hydrophobicity, resulting in their distinct biological activities [7]. As the main metabolites of ginsenosides, 20(S, R)-protopanaxadiol [PPD(S, R)] and 20(S, R)-protopanaxatriol [PPT(S, R)] are the structural basis response to a series of pharmacological effects of their
parent components [8]. Different orientations of the hydroxyl groups at C-20 provide stereoisomers, (R)-epimer and (S)-epimer, as shown in Fig. 1.

Based on the structural similarity of ginsenosides to steroid hormones, some pharmacological effects of ginsenosides may be mediated by binding to nuclear receptors, such as androgen receptor, estrogen receptors (ERs), glucocorticoid receptor, peroxisome proliferator-activated receptor γ, and progesterone receptor [9–12]. Nuclear receptor superfamily is a group of transcription factors that can be activated by the functional ligands, including 17β-estradiol, dexamethasone, fatty acids, etc [13–15]. As the main targets of phytoestrogens, ERs have been reported for their ability of binding several ginsenosides, including Re and Rb1 [9,16]. Nevertheless, Rb1 was observed to activate both ERα and ERβ in the absence of receptor binding, indicating that the estrogenic effect of Rb1 was mediated via a ligand-independent pathway [16]. In summary, ginsenosides might exert their estrogenic activities by either directly binding or indirectly activating ERs.

Although previous studies have confirmed the estrogenicity of several ginsenosides, however, the underlying mechanisms of their estrogenic effects are still largely unclear. In this work, PPD(S, R) and PPT(S, R), in vivo metabolites of ginsenosides, were assessed for their ability to bind and activate human estrogen receptor α (hERα). First, the recombinant hERα ligand-binding domain (hERα-LBD) was expressed in E. coli strain. The direct binding interactions of ginsenosides with hERα-LBD and their ERα agonistic potency were investigated by fluorescence polarization (FP) and reporter gene assays, respectively. Then, molecular docking was carried out to simulate the binding modes between ginsenosides and hERα-LBD in an attempt to reveal the structural basis for their agonist activities toward receptor protein.

2. Materials and methods

2.1. Materials

Coumestrol (CS), 17β-estradiol (E2), dimethylsulfoxide, and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and TCI (Tokyo, Japan). Lipofectamine 2000 transfection reagent, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco’s modified Eagle’s medium, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was purchased from HyClone (Logan, UT, USA). Ginsenosides PPD(S, R) and PPT(S, R) were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). The structures of the ginsenosides are shown in Fig. 1. All other reagents used were of analytical grade.

2.2. Preparation of glutathione S-transferase–hERα-LBD

The recombinant hERα-LBD (residues 282 to 595) was expressed as a fusion protein from the glutathione S-transferase (GST)-modified pGEX-4T-1 vector in Escherichia coli strain BL21(DE3)

Fig. 1. Structures of 20(S, R)-protopanaxadiol [PPD(S, R)] and 20(S, R)-protopanaxatriol [PPT(S, R)] investigated in this work. Different orientations of the hydroxyl groups at C-20 provide stereoisomers, (R)-epimer and (S)-epimer.
pLysS. The coding region was synthesized de novo and inserted into BamHI and XhoI restriction enzyme sites. The plasmid pGEX-4T-1-hERz-LBD was introduced into BL21(DE3)pLysS. Expression of the GST-tagged hERz-LBD protein was induced with 0.5 mM IPTG overnight at 20°C. The supernatant was loaded onto a glutathione-Sepharose affinity column. Homogeneity of the purified protein was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.3. Fluorescence polarization assay

The FP assay was performed by FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation at 355 nm and emission at 405 nm. The dissociation constant (K_{d,CS}) of CS with hERz-LBD was obtained from the saturable binding experiment. Subsequently, in the competitive binding assay, hERz-LBD (250 nM) and CS (10 nM) were mixed in a total volume of 290 μL and then various concentrations of ginsenoside (10 μL) were added. Each sample was subjected to the FP assay after being incubated for 2 h at room temperature. If the added ginsenoside could compete with the tracer for the protein binding site, it would displace CS from CS-hERz-LBD complex, resulting in a low polarization value. The decrease of polarization values upon addition of competitive compound was monitored and plotted as a function of the concentrations of ginsenoside.

The half maximal inhibitory concentration (IC_{50}) value (the concentration of ginsenoside that inhibited the binding of CS with hERz-LBD by 50%) was calculated from the competition curves fitted using a four parameter logistic equation: 

\[ Y = A - \frac{Y_0 - A}{1 + (X/IC_{50})^B} + D, \]

where Y and X correspond to the polarization value and the ginsenoside concentration, A and D are the polarization values at zero and an infinite concentration respectively, and B is the slope parameter [17]. The dissociation constant (K_{d}) of ginsenoside with hERz-LBD was calculated according to the relationship IC_{50}/[comestrol] = K_{d}/K_{d,CS}. Data analysis was performed using GraphPad Prism 5 (GraphPad Software, USA).

2.4. Construction of plasmids

The coding region for full-length hERz was obtained by polymerase chain reaction–based accurate synthesis and then cloned into the pcDNA3.1(+)- vector at restriction sites BamHI and XhoI to generate an expression plasmid pcDNA3.1(+)-hERz. The estrogen response element–luciferase (ERE-luc) reporter plasmid was constructed using a modified vector pGL6-CMV-TA-Luc containing the coding sequence for firefly luciferase. Two tandem repeats of the consensus ERE oligonucleotide (TGTAGTGTCACAGTGACCTGAT) upstream of the minimal TA promoter were inserted into the BmtNdeI site. The control plasmid pRL-TK was purchased from Promega (Madison, WI, USA).

2.5. Cell proliferation and cytotoxicity assay

MCF-7 breast cancer cells preserved in our lab were routinely cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg streptomycin. Cells were maintained at 37°C in a humidified 5% CO2 atmosphere. The cytotoxicity of each ginsenoside was measured using MTT assay. MCF-7 cells were seeded in a 96-well culture plate at a density of 1 × 10⁴ cells/well and allowed to acclimatize for 12 h, then treated with different concentrations of ginsenosides for 24 h. At the end of treatment, 20 μL of MTT was added and incubated for an additional 4 h. The formazan crystals were dissolved in 200 μL of dimethylsulfoxide. After shaking the plate for 10 min, cell viability was assessed by measuring the absorbance at 550 nm.

2.6. Luciferase reporter assay

A mixture of pcDNA3.1(+)–hERz, ERE-luc, and pRL-TK plasmids were prepared at a ratio of 1:10:1 firstly. After exposure to the transfection reagents and plasmids for 12 h, the cells were washed with phosphate buffered saline (PBS) and then incubated with a medium containing different concentrations of ginsenosides for an additional 24 h. The activities of both firefly and Renilla luciferase in cell lysates were determined using a Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instruction on a Tecan Infinite F200 PRO microplate reader. Firefly luciferase activity was normalized against that of Renilla in three independent experiments. Data analysis was performed using GraphPad Prism 5 (GraphPad Software, USA).

2.7. Molecular dynamics simulations

The crystal structure of hERz-LBD in complex with ortho-trifluoromethylphenylvinyl estradiol (EZT) was available in Protein Data Bank (PDB ID: 2P15) [18]. The initial structures of PPD (S, R) and PPT (S, R) were constructed using GaussView and optimized with Gaussian 09 using the B3LYP/6-31G(d) method. The molecular length and Connolly solvent-excluded volume of the tested compounds were calculated using AutoDockTools-1.5.6 and Chem3D Ultra 8.0, respectively. Automated docking calculations were carried out by AutoDockTools-1.5.6 to explore the binding modes between hERz-LBD and these compounds. The grid box was generated at the center of the active site. The size of the grid box should be adjusted according to the length of each compound. Based on the scoring function of AutoDockTools-1.5.6, the predicted binding energies (kcal mol⁻¹) were calculated. For each compound, 10 independent docking runs were performed, and the one with the lowest binding energy was chosen for analysis. The intermolecular interactions between hERz-LBD and ligands were visualized with the program PyMol.

| Compound | IC_{50} (μM) | K_{d} (μM) | Binding energy (kcal mol⁻¹) |
|----------|-------------|-------------|-----------------------------|
| PPD (S)  | 49.34       | 1260.64     | -11.68                      |
| PPD (R)  | 54.49       | 1392.22     | -11.51                      |
| PPT (S)  | 58.65       | 1498.51     | -11.26                      |
| PPT (R)  | 67.17       | 1716.19     | -11.12                      |

PPD (S, R), 20(S, R)-protopanaxadiol; PPT (S, R), 20(S, R)-protopanaxatriol.
3. Results and discussion

3.1. Characterization of the recombinant protein

For the subsequent FP assay, a soluble form of the hERα-LBD protein should be produced first. In this work, the recombinant protein was expressed in the IPTG-induced E. coli cultures and then purified by affinity chromatography on glutathione-Sepharose. As shown in Fig. 2, a soluble protein with an apparent molecular mass of 62.8 kDa was achieved, corresponding to the GST fusion protein containing the ligand binding domain of hERα. Unfortunately, an additional band (26.0 kDa) migrating below GST-hERα-LBD was observed in SDS-PAGE analysis, suggesting that truncation of the GST tag might happen during overexpression of the recombinant protein in BL21(DE3)pLysS. As GST protein does not bind to ERα [19], it can be speculated that the truncated tag may exert a negligible influence upon the ligand-ER interaction. Hence, although the soluble proteins produced in this work contain both intact GST-hERα-LBD and truncated GST, they are still suitable for in vitro binding assay.

3.2. Assessment of ginsenosides binding potency with hERα-LBD

Estrogen mimetics such as xenoestrogens and phytoestrogens regulate physiological functions in target tissues primarily by binding to steroid hormone receptors [20]. To test whether PPD(S, R) and PPT(S, R) mediate their biological effects through direct binding to hERα-LBD, the competitive binding assay was carried out by FP. CS, a plant-derived autofluorescent compound, was chosen as tracer for the ligand displacement experiment. It can compete with physiological estrogen (17β-estradiol) for binding to ER as well as stimulate the activity of reporter genes in the presence of its receptor protein [21]. As can be seen in Fig. 3, all the tested ginsenosides exhibited dose-dependent binding to hERα-LBD. Base on the Kd, CS of 255.50 nM determined in our previous work, the IC50 values and dissociation constants (Kd) of hERα-LBD

- **Table 2**
  | Compound | Length (Å) | CSEV (Å3) | Hydrogen bonds | Estrogenicity |
  |----------|------------|-----------|----------------|--------------|
  | EZT      | 11.782     | 391.5     | Glu353, Leu387, His394, H2O | Agonist |
  | PPD(S)   | 14.709     | 483.2     | Glu353          | Agonist |
  | PPD(R)   | 15.265     | 483.9     | Glu353, His524 | Agonist |
  | PPT(S)   | 14.948     | 488.7     | Glu353          | Agonist |
  | PPT(R)   | 15.258     | 489.6     | Glu353, His524 | Agonist |

EZT, ortho-trifluoromethylphenylvinyl estradiol; PPD, protopanaxadiol; PPT, protopanaxatriol.

![Fig. 3. Competitive binding of PPD(S, R) and PPT(S, R) to hERα-LBD. Results are given as means ± SEM of three independent experiments.](image)

hERα-LBD, human estrogen receptor α-ligand-binding domain; PPD (S, R), protopanaxadiol; PPT (S, R), protopanaxatriol.

SEM, standard error of the mean

![Fig. 4. The hERα activation potency determined by ERE-luciferase reporter gene assay in MCF-7 cells. (A) Of E2. (B) Of ginsenosides. Results are given as means ± SD of three independent experiments.](image)

ERE, estrogen response element; hERα, human estrogen receptor α; PPD, protopanaxadiol; PPT, protopanaxatriol; SD, standard deviation.

![Fig. 5. Superimposition of the docking poses of different ligands in the hERα-LBD. EZT, blue; PPD(S), yellow; PPD(R), cyan; PPT(S), magenta; PPT(R), orange.](image)

hERα-LBD, human estrogen receptor α-ligand-binding domain; PPD, protopanaxadiol; PPT, protopanaxatriol.
for PPD(S, R) and PPT(S, R) were measured and illustrated in Table 1. The $K_d$ values of the tested ginsenosides are in the range of 1260.64 $\mu$M to 1716.19 $\mu$M, reflecting their moderate affinities for hER$\alpha$-LBD. With respect to all these compounds, the binding affinities are in the order of PPD(S) > PPD(R) > PPT(S) > PPT(R). In summary, all the ginsenosides investigated in this work can bind to hER$\alpha$-LBD as the functional ligands, resulting in the activation of ER which may in turn influence the regulation of various physiological functions.

3.3. Biological effect of ginsenosides on hER$\alpha$ activity

For subsequent luciferase reporter gene assay, the cytotoxicity of the tested compounds should be assessed by MTT assay first. The hER$\alpha$ activity in MCF-7 cells was measured under the exposure of noncytotoxic concentrations of ginsenosides. This allowed us to ensure that changes in hER$\alpha$ activity were due to ginsenosides treatment but not a secondary effect of cell death. In the present work, MCF-7 cells were transiently cotransfected with plasmids of

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**Fig. 6.** Computational docking of EZT, PPD(S, R) and PPT(S, R) to hER$\alpha$-LBD. (A) The hydrophobic binding pocket of hER$\alpha$-LBD to stabilize ligands. (B) The profiles of ligand-hER$\alpha$-LBD interaction. Red sphere, water molecule; red dashed lines, hydrogen bonds.

hER$\alpha$-LBD, human estrogen receptor $\alpha$-ligand-binding domain; EZT, ortho-trifluoromethylphenylvinyl estradiol; PPD, protopanaxadiol; PPT, protopanaxatriol.
pcDNA3.1(+)–hERz and ERE-luc to investigate the activation effect of ginsenosides on the hERz pathway. The cells were also cotransfected with a plasmid (pRL-TK) encoding Renilla luciferase controlled by a constitutively active promoter to correct for the difference in both transfection and harvest efficiencies between experiments. The fold of hERz activation was expressed as the ratio of firefly to Renilla luciferase activity (Fluc/Rluc). The transfected cells were treated with the tested compounds over a range of concentrations. After incubation for 24 h, luciferase activity was measured.

As shown in Fig. 4A, the in vitro transient transfection system in MCF-7 cells is well established and suitable for evaluating the effects of ginsenosides on hERz activation. Luciferase reporter gene assay showed that hERz was activated by PPD(S, R) and PPT(S, R) in a dose-dependent manner with a maximal of four-fold increase (Fig. 4B). Furthermore, it can be observed that the order of activation potency for ginsenosides is good agreement with their binding affinities with hERz-LBD.

3.4. Structural basis for ginsenosides binding with hERz-LBD

Major ginsenosides are a series of dammarane-type triterpene homologues that share a common hydrophobic four-ring steroid-like structure. They can be categorized into two groups according to the aglycone structure: PPD and PPT types. The former has hydrophilic sugar moieties attached at the positions of C-3 and/or C-20 (such as Rb1, Rb2, Rc, and Rd), while the latter has hydrophilic sugar moieties attached at the positions of C-3, C-6, and/or C-20 (such as Re and Rg3) [22,23]. As the main metabolites of ginsenosides, PPD(S, R) and PPT(S, R) were observed to bind and activate hERz in this work. Then, to elucidate the structure–activity relationship with a focus on hydroxyl groups located in different positions and the stereoselectivity of 20(S) and 20(R), the binding modes between ginsenosides and hERz-LBD were simulated by molecular docking.

Based on the crystal structure of human ERz in complex with 17β-estradiol (Ez) [24], the ligand-binding pocket (LBP) with a probe accessible volume of approximately 450 Å³ can hardly accommodate PPD(S, R) or PPT(S, R) (~485 Å³), as summarized in Table 1. As shown in Fig. 5 and Table 2, the skeleton length of ginsenosides (~15 Å) is well matched by the LBP. They completely fit into the cavity without disrupting the coactivator binding site, known as a transcriptional activation function (AF-2). Interestingly, PPD(S, R) and PPT(S, R) all adopt an agonist conformation similar to that of E2 (Fig. 5), a potent agonist ligand.

Molecular docking results suggested that the stereostructure of the C-20 hydroxyl group exerts significant influence on the hydrogen bonding and hydrophobic interactions. As shown in Fig. 6, 20(R)-ginsenosides form a hydrogen bond with His524 in H11. However, the hydrogen bond between this amino acid site and 20(S)-ginsenosides has not been observed. Thus, the higher affinities of 20(S)-ginsenosides may be associated with an increase in the number of hydrophobic contacts compared with their 20(R) counterparts. Besides, the C-3 hydroxy group locates in the active site of hERz-LBD and makes a hydrogen bond interaction with Glu353 in H3. On the other hand, with a hydroxyl group at C-6, the steric hindrance for PPT to bind to hERz-LBD increases, and thus significantly reducing their binding potency compared to PPD, as shown in Table 1. It has been reported that the PPD-type ginsenosides generally exert more potent biological effects than those of the PPT-type [25], which is confirmed in this work.

Furthermore, the order of the calculated binding potency for ginsenosides with hERz-LBD is consistent with their experimentally determined binding affinities (Table 1). As shown in Fig. 7, comparison of the docking scores versus the IC50 values yields a good correlation (R² = 0.94), indicating that molecular dynamics simulations can potentially be applied for predicting the binding potency of novel bioactive compounds toward target receptors.

4. Conclusion

In this work, the relationship between ginsenoside structures and their estrogenicity was assessed by a combination of in vitro and in silico analysis. ERE-luc reporter gene assay showed that PPD(S, R) and PPT(S, R) were capable of activating hERz in transiently transfected MCF-7 cells. FP competitive binding assay suggested that these ginsenosides exert estrogenic activities through binding to hERz-LBD. Furthermore, molecular dynamics simulations were performed to elucidate the underlying mechanisms of their estrogenic effects. In conclusion, as the main metabolites of ginsenosides, PPD(S, R) and PPT(S, R) display apparent functional properties comparable to those of ER agonists. This work may provide insight into chemical and pharmacological approaches in the discovery and evaluation of novel therapeutic agents that target ER.

Conflicts of interest

All authors have no conflicts of interest to declare.

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