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Glyceollin I enantiomers distinctly regulate ER-mediated gene expression

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ABSTRACT

Glyceollins are pterocarpan phytoalexins elicited in high concentrations when soybeans are stressed. We have previously reported that the three glyceollin isomers (GLY I–III) exhibit antiestrogenic properties, which may have significant biological effects upon human exposure. Of the three isomers, we have recently shown that glyceollin I is the most potent antiestrogen. Natural (−)-glyceollin I recently was synthesized along with its racemate and unnatural (+) enantiomer. In this study, we compared the glyceollin I enantiomers' ER binding affinity, ability to inhibit estrogen responsive element transcriptional (ERE) activity and endogenous gene expression in MCF-7 cells. The results demonstrated similar binding affinities for both ERα and ERβ. Reporter gene assays in MCF-7 cells revealed that while (+)-glyceollin I slightly stimulated ERE transcriptional activity, (−)-glyceollin I decreased activity induced by estrogen. Co-transfection reporter assays performed in HEK 293 cells demonstrated that (+)-glyceollin I increased ERE transcriptional activity of ERα and ERβ with and without estrogen with no antiestrogenic activity observed. Conversely, (−)-glyceollin I decreased the activity of both ER subtypes stimulated by estradiol demonstrating potent antiestrogenic properties. Additionally, each Gly I enantiomer induced unique gene expression profiles in a PCR array panel of genes commonly altered in breast cancer.

1. Introduction

Flavonoids are a class of plant secondary metabolites and they can be classified into three categories: flavonoids, isoflavonoids (isoflavones) and neoflavonoids. Flavonoids have been extensively studied and are known for their health benefits provided against cancer and heart disease [1–5]. Isoflavones are a distinctive subclass of flavonoids. These compounds play important roles in plant and animal health [6]. Studies have shown that regular consumption of soy isoflavones has been associated with reduced risk of breast and uterine cancer [7–9]. While the mechanism of action of isoflavones and their derivatives are continuously being studied, the details of their ability to alter estrogen effects remain unclear. Many of the more complex isoflavones such as the pterocarpan phytoalexins are produced in response to fungal pathogens and other stresses [10]. Pterocarpans are the second largest group of natural isoflavones which have received considerable interest for their medicinal properties. The biological properties of the pterocarpans range from stress response and antimicrobial activity in plants to hormonal regulating and anticancer activity in mammalian systems [11–16]. Our research group has focused on one particular family of pterocarpans produced in soybean: the glyceollins.

Glyceollins are pterocarpan phytoalexins which are produced in soybean tissue when exposed to stress or elicitors (abiotic or biotic) [17–19]. The mixture of glyceollins produced consists of

Abbreviations: 4-OH-Tam, 4-hydroxytamoxifen; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen responsive element; GLY, glyceollin; ICI 182,780, Fulvestrant; PgR, progesterone receptor; SERM, selective estrogen receptor modulator; 3ERT, human estrogen receptor-alpha ligand-binding domain in complex with 4-hydroxytamoxifen.

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three major isomers (I–III). The three glyceollin isomers have similar core structures to coumestrol and are derived from the parent isoflavone daidzein through a series of pterocarpan intermediates. Previously we have compared the glyceollin mixture to genistein and daidzein and found that the glyceollin mixture has a greater inhibition of estradiol effects on proliferation and estrogen receptor signaling in breast cancer cells [20]. There were no estrogenic effects observed with the glyceollins *in vitro*, in contrast to other major soy isoflavones. Both *in vitro* and *in vivo* studies have indicated that the glyceollins exhibit marked antiestrogenic activity in various tissues and even demonstrate anticancer properties [20–22]. Recently, we examined the molecular effects of soy phytoalexin, glyceollins, on human prostate cancer cells, LNCaP and found that glyceollin inhibited LNCaP cell growth in a similar manner to that of the soy isoflavone genistein. We also found that glyceollin treatment led to down-regulated mRNA levels for androgen responsive genes and this response appeared to be mediated through modulation of an estrogen but not androgen-mediated pathway [23]. Glyceollin exerted multiple effects on several different cell systems and may be considered a cancer therapeutic agent.

The three glyceollin isomers have similarities in their core ring structure (rings A–C) but differ in their size and position of the last ring formed attached at ring A during the last step of the biosynthesis. This structural difference could lead to different biological effects; therefore, we isolated the individual glyceollin isomers in an attempt to identify the specific isomer responsible for the marked antiestrogenic activity. Through a series of biological assays we have identified glyceollin I as the active antiestrogenic isomer of the glyceollin mixture [24]. Zimmernamm et al. demonstrated that among the three glyceollin isomers, glyceollin I displayed the highest affinity for ERα with an IC_{50} value of 1.68 μM. Through ligand-receptor modeling (docking studies) it appears that glyceollin I, but not isomers II and III, can dock reasonably to the ERα ligand-binding cavity suggesting a unique type II antiestrogenic binding mode [24]. We further compared the effects of glyceollin I to antiestrogens 4-hydroxytamoxifen (4-OH-Tam) and fulvestrant (ICI 182,780) in MCF-7 breast cancer cells and BG-1 ovarian cancer cells on 17β-estradiol (E_2) stimulated expression of progesterone receptor (PgR) and stromal derived factor 1-α (SDF-1). We found that glyceollin I inhibited ER-mediated gene expression and cell survival [24]. Based on this information, glyceollin I may have potential as an alternate form of treatment for postmenopausal women with breast cancer who eventually may become resistant to tamoxifen therapy.

To further investigate the antiestrogenic properties of glyceollin I and to consider its effectiveness as a breast cancer treatment, the synthesis of glyceollin I was initiated. The first total synthesis of glyceollin I as its natural (−)-enantiomer having 6aS, 11aS stereochemistry was accomplished by Khupse and Erhardt, who also prepared the unnatural (+)-enantiomer as well as the racemate [25]. Glyceollin I possesses two asymmetric center carbons at the 6a and 11a positions, which could result in four stereoisomers. However, the one enantiomer produced by anabolic process from isoflavones in plants is known to be the (−)-glyceollin I which bears only the *cis* (6aS, 11aS) stereochemistry [26]. Computational studies suggest that the 6a, 11a-*cis* ring junction is also energetically more favored over the *trans* [27]. The majority of the known natural pterocarpans possess the 6a, 11a-*cis* configuration [28–30] and the (−)-glyceollin I is the natural enantiomer produced when soy beans are placed under stress.

A number of reports have demonstrated enantiomer-specific binding and showed differences in the biological activity of enantiomers toward the estrogen receptor such as diethylstilbestrol (DES) [31–37], diarylpropiolnitrile (DPN) [38], dichlorodiphenyltrichloroethane (DDT) [39], and equol [40–43]. Setchell et al. showed that equol produces two enantiomers which display different binding affinities for the estrogen receptors and produced distinct biological activities [40,41]. In this report, we evaluate for the first time the ERα and ERβ binding affinities, transcriptional activity, ER docking and gene expression analysis of (+)-glyceollin I and (−)-glyceollin I.

2. Experimental

2.1. Chemicals

4-Hydroxytamoxifen (4-OH-Tam) and 17β-estradiol (E_2) were purchased from Sigma (St. Louis, MO). Fulvestrant (ICI 182,780) was purchased from Tocris Bioscience (Ellisville, MO). Racemic, (+)- and (−)-glyceollin I were prepared by a novel synthetic process by Khupse and Erhardt at the University of Toledo [25].

2.2. Cell culture

Human cancer cell lines derived from breast, MCF-7 (ER-positive cells) and human embryonic kidney, HEK 293 were cultured in 75 cm^2 culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Co.) supplemented with 10% fetal bovine serum (FBS) (Life Technologies Inc., Gaithersburg, MD), basic minimum MEM essential (50×, Invitrogen Co.) and MEM non-essential (100×, Invitrogen Co.) amino acids, sodium pyruvate (100×, Invitrogen Co.), antimycotic–antibiotic (10,000 U/mL penicillin G sodium; 10,000 μg/mL streptomycin sulphate; 25 μg/mL amphotericin B as Fungizone®), and human recombinant insulin (4 mg/mL, Invitrogen Co.). The culture flasks were maintained in a tissue culture incubator in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. For estrogen studies, cells were washed with PBS 3 times and grown in phenol-red free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5% CS-FBS).

2.3. ER binding assays (ER α and β)

Receptor binding determinations of (+)-glyceollin I and (−)-glyceollin I enantiomers were achieved using the method of Bolger et al. [44] as was applied by Burow et al. [20]. In this method, recombinant ER is in equilibrium with a fluorescent ligand (ES2) and a concentration of the competitor ((+)- and (−)-glyceollin I enantiomers). The relative displacement of the ES2 is measured as a change in polarization anisotropy. Serial dilutions of competitors ((+)- and (−)-glyceollin I enantiomers and estradiol) were prepared from DMSO stock solutions in screening buffer at the desired concentrations. The ER and ES2 were combined with each competitor aliquot to a final concentration of 2 nM ER and 3 nM ES2, respectively. In addition, both a no binding control (ER + ES2, equivalent to 0% competitor inhibition) and a 100% binding control (only free ES2, no ER, equivalent to 100% competitor inhibition) were prepared. All competitor and controls were prepared in duplicate within a binding experiment. After 2 h incubation at room temperature, the anisotropy value for each sample and control was measured using the Beacon 2000. Anisotropy values were converted to percent inhibition using the following formula: $I_{\%} = (A_0 - A)/(A_0 - A_{100}) \times 100$, where $I_{\%}$ is the percent inhibition, A_0 is 0% inhibition, A_{100} is 100% inhibition, and A represents the observed value. This conversion to percent inhibition makes the data more intuitive and normalizes the experiment-to-experiment differences in the range of anisotropy values. The percent inhibition versus competitor concentration curves were analyzed by nonlinear least-squares curve fitting (Prism 5.0a, GraphPad Software, San Diego, CA, USA, www.graphpad.com) to yield IC_{50} values (the concentration of competitor needed to displace half of the bound ligand). To compare binding affinities of the test compounds to those reported in

the literature, IC_{50} values were converted to relative binding affinities (RBA) using E_2 as a standard. The E_2 RBA was set equal to 100RBA = (IC_{50}/IC_{50} of E_2) × 100.

2.4. ERE-luciferase assay

ER(2)-luc, pcDNA-ERα and pcDNA-ERβ expression plasmids have been previously described [45]. MCF-7 breast cancer cells were plated in 24-well plates at a density of 5×10^5 cells/well in 5% charcoal-stripped phenol-red free media and allowed to attach overnight. After 18 h, cells were transfected with 0.3 μg of ER(2)-luc plasmid (Panomics) for 6 h according to the manufacturers protocol using Effectene (Qiagen) and treated with vehicle DMSO, (+)-glyceollin I and (−)-glyceollin I (0.01–10 μM) (all with and without estrogen) overnight. Media were removed and cells were lysed with reporter lysis buffer. Relative light units (RLUs) were measured in an Opticomp II luminometer (MGM Laboratories) using luciferase reagent (Promega).

HEK 293 cells were seeded into 24-well plates at a density of 5×10^5 cells/well and allowed to attach overnight in 5% charcoal-stripped phenol-red free media overnight. Cells were transfected with 0.2 μg ER(2)-luc plasmid (Panomics), 0.2 μg pcDNA3.1B-ERβ or 0.2 μg pcDNA3.1B-ERα plasmids and the next day using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. After a 6 h transfection, cells were treated with compounds (DMSO, (+)-glyceollin I and (−)-glyceollin I (0.01–10 μM) (all with and without estrogen)) overnight. On the following day, the cells were lysed with 150 μl of the M-Per mammalian extraction reagent (Pierce). One hundred microliters of cell extract were assayed using the Bright-glo luciferase assay substrate (Promega) and determined in a Berthold AutoLumat Plus luminometer. The data are the results of at least three independent experiments with three replicates each. Data were summarized as the mean ± standard error of the mean (SEM) using the Graph Pad Prism V.4 software program (GraphPad Software Inc.). Analysis of variance models was employed to compare relative ERE transcriptional reporter activity between controls versus different doses of glyceollin I enantiomers with and without E_2 stimulation. A Tukey post-test was performed to compare differences between groups, where a p value < 0.05 was considered significant. All treatment groups compared with estrogen are designated as "a" whereas all treatment groups compared with control are designated as "b." Results are expressed as the mean unit ± SEM (***$p < 0.001$; **$p < 0/01$; and *$p < 0.05$).

2.5. Gene superarrays

MCF-7 cells were seeded into 75 cm^2 flasks in DMEM media supplemented with 5% fetal bovine serum. On the following day media were replaced with phenol-red free DMEM supplemented with 5% charcoal-stripped serum for 2 days. Cells were treated with DMSO (vehicle), 1 nM 17β-estradiol, 10 μM (+)-glyceollin I and (−)-glyceollin I. Total RNA was extracted. Each array profiles the expression of a panel of 96 genes. For each array, 4 μg RNA was reverse transcribed into cDNA in the presence of gene-specific oligonucleotide primers as described in the manufacturer's protocol. cDNA template was mixed with the appropriate ready-to-use PCR master mix, equal volumes were aliquoted to each well of the same plate, and then the real-time PCR cycling program was run. Quantitative RT-PCR was performed using manufacturer's protocols for the RT^2 Profiler^{TM} PCR Array (Human Breast Cancer and Estrogen Receptor Signaling Superarray, Gaithersburg, MD, USA). Relative gene expressions were calculated by using the $2^{-\Delta\Delta Ct}$ method, in which Ct indicates the fractional cycle number where the fluorescent signal reaches detection threshold. The 'delta–delta' method [46] uses the normalized ΔCt value of each sample, calculated using a total of five endogenous control genes (18S rRNA, HPRT1, RPL13A, GAPDH, and ACTB). Fold change values are then presented as average fold change = $2^{-(\text{average } \Delta\Delta Ct)}$ for genes in treated relative to control samples. Clinical variables were characterized using descriptive statistics, and the statistical significance of differences in gene expression between groups was calculated using Student's t-test.

2.6. Molecular modeling

The structures of (+)-glyceollin I and (−)-glyceollin I enantiomers were converted to unique SMILE strings with ChemDraw (CambridgeSoft, cambridgesoft.com) and then converted to a 3D structures using MOE 2008.10 (Chemical Computing Group, Montreal, Quebec, Canada, H3A 2R7). The initial 3D models were then optimized in MOE using the MMFF94 force field with the conjugated gradient method using a termination of 0.005 kcal/mol. The (+)-glyceollin I and (−)-glyceollin I enantiomer models were added to a database containing an optimized model of 4-OH-tamoxifen. Docking and scoring of the (+)-glyceollin I and (−)-glyceollin I enantiomers was performed using the crystal structure of the human estrogen receptor-alpha ligand-binding domain in complex with 4-OH-tamoxifen (pdb: 3ERT, antagonist configuration) and the docking function of MOE. The A chain, including associated waters, was extracted from the 3ERT crystal structure and then hydrogens were added and optimized for docking with the Protonate 3D function of MOE. The models of the (+)-glyceollin I and (−)-glyceollin I enantiomers and 4-OH-tamoxifen were then docked into the 3ERT Chain A model with MOE dock using the Triangle Matcher placement (default settings), London dG rescoring 1, Force field refinement (default settings) and London dG rescoring 2. This docking procedure was previously defined in this study as the MOE docking method that produced the best RMSD (0.758) replacement of the 4-OH-tamoxifen model into this crystal structure. Of up to 30 scoring poses obtained for each of the three models docked into the tamoxifen induced, antagonist form of the estrogen receptor alpha, less than 5 were found to have favorable docking scores (S) and reasonable poses in the binding cavity (occupy significant portion of the binding pocket). Of these, the best scoring poses were selected to depict possible ER binding modes for the (+)-glyceollin I and (−)-glyceollin I enantiomers.

3. Results

3.1. Relative affinity of glyceollins I enantiomers for ERα and ERβ

In an attempt to understand the biological activity of the synthesized glyceollin enantiomers, we examined the ability of (+)-glyceollin I and (−)-glyceollin I enantiomers to bind to ERα and ERβ by using a competitive binding assay with fluorescent detection (Fig. 1). Analysis of the competition binding curve yielded IC_{50} values, (concentration of unlabeled ligand required to displace 50% of the tracer from the ERα and ERβ). A displacement of 50% E_2 bound to each receptor subtype was quantitated to determine the affinity of the two enantiomers for ERα and ERβ (Fig. 1). The two glyceollin I enantiomers displayed similar binding affinities for ERα and ERβ. For ERα, the IC_{50} value of (−)-glyceollin I was 3.03 μM and the (+)-glyceollin I was 2.38 μM. For ERβ, the IC_{50} value of (−)-glyceollin I was 4.60 μM and the (+)-glyceollin I was 7.01 μM. These results show that both glyceollin I enantiomers bind similarly to the estrogen receptor subtypes and that the glyceollin I enantiomers do not possess enantio-selectivity toward either estrogen receptor subtype.

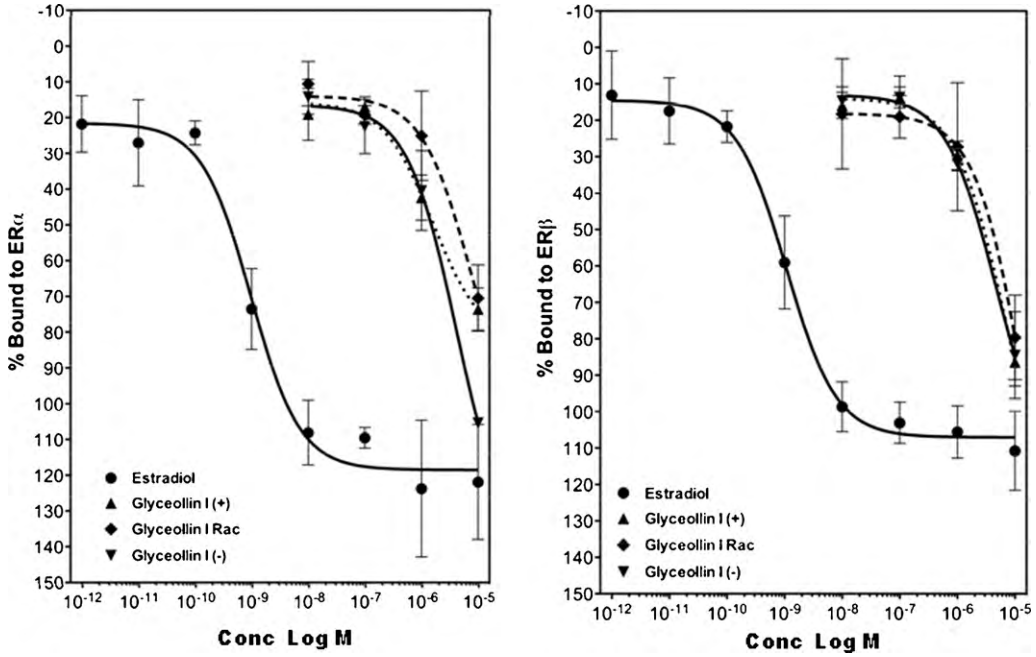

Fig. 1. Relative binding affinity of glyceollin I enantiomers. Competition binding curves of the (+)-glyceollin and (−)-glyceollin I enantiomers binding to ERα (left) and ERβ (right). Increasing concentrations of the (+)-glyceollin and (−)-glyceollin I enantiomers were added to the ERα and ERβ complex and compared to E_2. Data points and error bars represent the mean ± SEM of at least three experiments ($n = 3$) for each concentration tested ($p < 0.05$).

3.2. *Effect of the (+)- and (−)-glyceollin I on ER transcriptional activation in MCF-7 cells*

In this study, we examined the ER transcriptional activity induced by glyceollin I enantiomers using an estrogen responsive element (ERE) based luciferase reporter gene assay. Initially, we examined the potential antiestrogenic properties of the glyceollin I enantiomers by using MCF-7 breast cancer cells possessing a system expressing both endogenous ERα and ERβ. The results from the MCF-7 cells treated with the (−)-glyceollin I enantiomer showed a decrease in ERE transcriptional activity when compared to control; whereas the (+)-glyceollin I seemed to slightly stimulate ERE transcriptional activity in a dose-dependent manner (Fig. 2). The (−)-glyceollin I plus E_2 resulted in a dose-dependent decrease in ERE transcriptional activity at concentration at or above 1 μM. In contrast, the (+)-glyceollin I plus E_2 did not cause a significant change in ER-mediated transcriptional activity. These results support our earlier findings which suggest that (−)-glyceollin I exhibits antagonist and antiestrogenic activity.

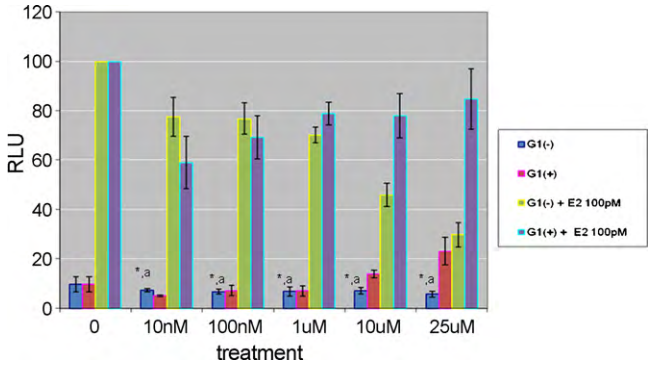

Fig. 2. Effects of (−)-glyceollin I and (+)-glyceollin I on ERE transcriptional activity in MCF-7 cells. MCF-7 cells were transiently transfected with pGL2-ERE2x-TK-luciferase plasmid. After a 5 h transfection, cells were treated with DMSO, E2, (+)-glyceollin I, (−)-glyceollin I, E2 + (+)-glyceollin I and E2 + (−)-glyceollin I and incubated for 18 h. Data are represented as relative light units (RLUs) normalized to E_2 (100 ± SEM). The values are the means and the SEM of triplicates from a single experiment and representative for at least three independent experiments. [a]Significant difference from E_2, $^*p < 0.05$.

3.3. *Effect of the (+)- and (−)-glyceollin I on ERα-dependent ERE transcriptional activation in HEK 293 Cells*

To determine whether the glyceollin I enantiomers exhibit enantiomer-specific activation of the ERE-dependent transcription through ERα or ERβ, we transiently transfected HEK 293 cells with an ERE-luciferase reporter construct in addition to ERα or ERβ. We chose HEK 293 cells (human embryonic kidney) because they lack endogenous ERα and ERβ. Estrogen responsive reporter gene assays were performed using HEK 293 cells treated with (+)- and (−)-glyceollin I with and without estrogen and were examined for ERE transcriptional activity. Results demonstrated that the (+)-glyceollin I increased ERE transcriptional activity in a dose-dependent manner with both ERα (Fig. 3A) and ERβ (Fig. 3B). Treatment with (+)-glyceollin I ranging from 0.1 to 10 μM enhanced ERα-mediated transcriptional activity to levels that were 25–83% above the control and the ERβ-mediated transcriptional activity to levels that were 30–153% above the control. (+)-Glyceollin I at 10 μM significantly increased ERα and ERβ transcriptional activity and also stimulated the estrogen induced transcriptional activity for both ER subtypes (Fig. 4A and B). These results suggest that unlike the natural enantiomer, the (+)-glyceollin I may possess estrogen agonist properties. In contrast, (−)-glyceollin I decreased the transcriptional activity in a dose-dependent manner below the control and blocked estrogen stimulation of ERα (Fig. 4A) and ERβ (Fig. 4B). Treatments with (−)-glyceollin I ranging from 0.1 to 10 μM abolished ERα-mediated transcriptional activity to levels that were 22–94% below the control (Fig. 3A). ERβ-mediated transcriptional activity treatment with (−)-glyceollin I at 0.1 and 1 μM were 51 and 31% above the control; whereas treatment at 10 μM was 79% below control (Fig. 3B). (−)-Glyceollin I at 10 μM significantly decreased ERα and ERβ transcriptional activity 94% and 79%, respectively compared to control. These results further demonstrated that the (−)-glyceollin I enantiomer exhibits antiestrogenic

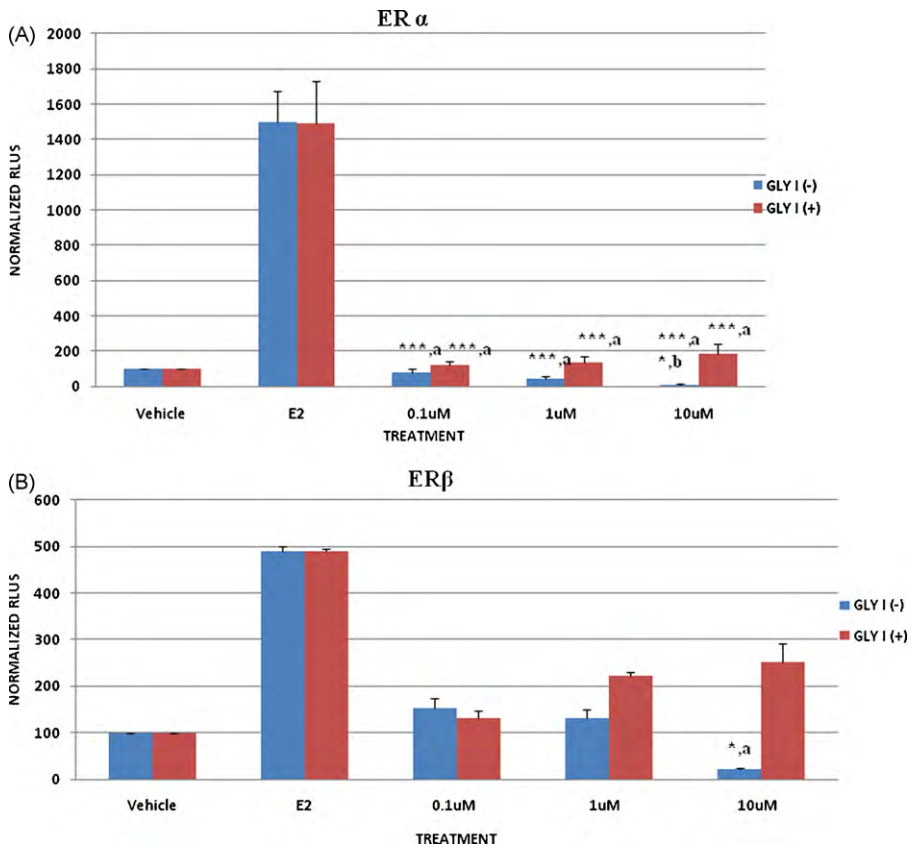

Fig. 3. Effects of (−)-glyceollin I and (+)-glyceollin I on ERE transcriptional activity in HEK 293 cells transfected with ERα and ERβ. HEK 293 cells were transiently transfected with ERE-luciferase plasmid, pcDNA3.1B-ERα (A) or pcDNA3.1B-ERβ (B). After a 5 h transfection, cells were treated with DMSO, E2, (+)-glyceollin I and (−)-glyceollin I and incubated for 18 h. Data are represented as relative light units (RLUs) normalized to untreated vector vehicle (100 ± SEM). The values are the means and the SEM of triplicates from a single experiment and representative for at least three independent experiments. [a]Significant difference from E_2, ***$p < 0.001$; [b]significant difference from vector vehicle, *$p < 0.05$.

and antagonistic behavior similar to that observed previously for the elicited glyceollin mixture and from our studies that utilized the natural glyceollin I as the single separated isomer.

3.4. Effects of the (+)- and (−)-glyceollin I on gene expression

To further examine the biological differences between the two glyceollin I enantiomers, we investigated the gene expression by performing a superarray analysis using an extensive panel of genes which are commonly altered in breast cancer and estrogen signaling. We chose to treat the MCF-7 cells with 1 nM E_2 and with 10 μM of the individual glyceollin I enantiomers. Total RNA was extracted, quantitated and a real-time PCR array was performed. The superarray showed that the glyceollin I enantiomers exhibited differences in their pattern of gene expression (see Table 1). As expected, E_2 treatment caused a 20.45-fold increase in PgR gene expression, whereas the (+)-glyceollin I treatment caused a 5.10 increase and the (−)-glyceollin I treatment caused a −3.58-

Table 1
Superarray analysis of genes altered by estradiol, (+)-glyceollin I and (−)-glyceollin I enantiomers treatment.

Gene name	Gene symbol	E_2 treatment	Glyceollin I (−) treatment	Glyceollin I (+) treatment
Progesterome receptor	PGR	**20.45**	−3.54	5.10
BCL2-associated athanogene	BAG1	−4.49	−4.96	−3.00
Androgen receptor	AR	−2.04	−3.17	−1.23
Trefoil factor 1	TFF1 or pS2	3.12	1.23	2.00
B-cell CLL/lymphoma 2	BCL2	1.15	−4.89	−1.09
Cyclin A1	CCNA1	3.85	3.23	1.40
Clusterin	CLU	−3.53	−2.32	1.29
Receptor tyrosine kinase erb B-2	ERBB2	−5.17	−1.18	−1.08
Fos-related antigen 1	FOSL1	3.39	4.69	1.01
Nerve growth factor receptor (TNFR superfamily, member 16)	NGFR	1.86	**14.20**	1.67
Gamma-aminobutyric acid receptor subumit pi precursor	GABRP	−26.93	−11.59	−4.06
Kallikrein-5 precursor	KLK5	3.31	2.12	−1.20
Keratin	KRT19	−1.22	−3.68	−1.40
Mucin-1 precursor	MUC1	−3.19	−4.39	−2.42
Plas minogen actovator inhibitor 1 precursor	SERPINE 1	1.28	2.17	4.38
Large neutral amino acids transporter small submit 1	SLC7A5	1.33	3.74	2.51
Tumor necrosis factor, alpha-induced protein 2	TNFAIP2	−1.61	−6.27	−5.60

Gene expression of select genes from superarray analysis which exhibited significant changes in expression with E_2 treatment, (+)-glyceollin I and (−)-glyceollin I treatment is shown. Numbers in bold color indicate fold changes in gene expression greater than 2 with red indicating up-regulation and blue indicating down-regulation.

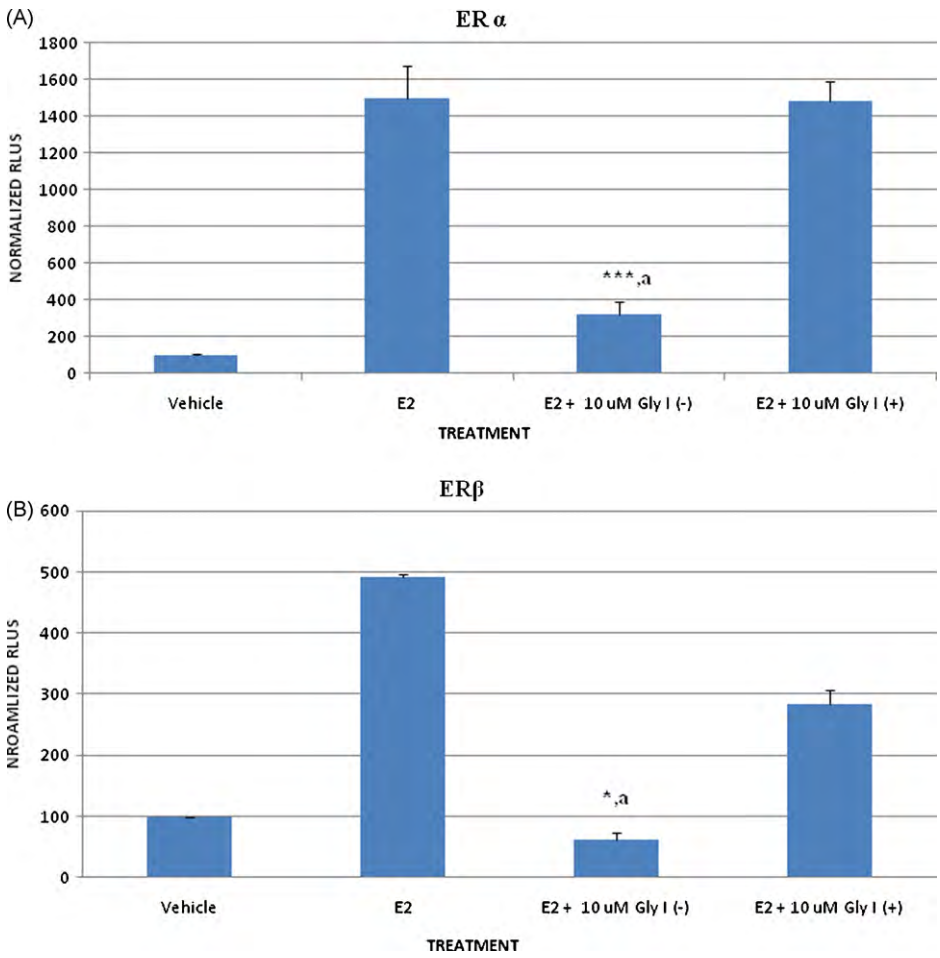

Fig. 4. Effects of (−)-glyceollin I and (+)-glyceollin I on E_2 stimulation on ERE transcriptional activity in HEK 293 cells transfected with ERα and ERβ. HEK 293cells were transfected with ERE-luciferase plasmid, pcDNA3.1B-ERα (A) or pcDNA3.1B-ERβ (B). After a 5 h transfection, cells were treated with DMSO, E2, (+)-glyceollin I and (−)-glyceollin I at 10 μM with estrogen and incubated for 18 h. Data are represented as relative light units (RLUs) normalized to untreated vector vehicle (100 ± SEM). The values are the means and the SEM of triplicates from a single experiment and representative for at least three independent experiments. [a]Significant difference from E_2, $^{***}p < 0.001$ and $^{*}p < 0.05$.

fold decrease in PgR gene expression. We identified several genes upregulated by E_2 alone: CCNA1, FOSL1, KLK5, SERPINA3, TFF1, and PgR. The following genes were down-regulated by E_2 alone: BAG1, CLU, ERBB2, GABRP, and MUC1. We also identified two genes uniquely upregulated by E_2 and the (−)-glyceollin I, CCNA1 and FOSL1. Nerve Growth Factor Receptor (NGFR), a member of the tumor necrosis factor receptor (TNFR) superfamily, was one gene which was uniquely upregulated by the (−)-glyceollin I. The latter is similar to the results previously shown by Zimmernamm et al. [24]. When comparing the (+)-glyceollin 1, (−)-glyceollin I and E_2, NGFR exhibited a differential pattern of expression. (−)-Glyceollin 1 caused a 14.20-fold increase in NGFR whereas the (+)-glyceollin 1 and estrogen did not cause a significant change in expression. (−)-Glyceollin I and E_2 caused a −26.93- and −11.59-fold decrease in GABRP; whereas with the (+)-glyceollin 1 this gene was only slightly down-regulated.

3.5. Docking glyceollin I enantiomers to estrogen receptor

Both of the glyceollin I enantiomers were docked to the 3ERT, OHT-Tam induced antagonist form of ERα. Resulting binding poses for each utilize the D-ring phenolic ring in the ligand-binding pocket the same way as the phenolic rings of 4-OH-Tam and E_2 H-bond to Arg 395, Glu 353 and H_2O (Fig. 5E and F). While the docked ERα binding mode of both glyceollin I enantiomers utilize the phenolic D-ring to anchor in the steroid A-ring site of the binding cavity, the remainder of their conjugated ring system extends out of the binding pocket in the same channel used by the aryl amine side chain of 4-OH-Tam to displace helix-12 into the antagonist configuration of ER (shown in Fig. 5A and B). This binding mode is in accord with the observed glyceollin I ER antagonist activity. Alternatively, the binding modes of the glyceollin I enantiomers proposed from the docking studies present different pterocarpan ring configurations and receptor interactions for each individual enantiomer. The (−)-glyceollin I ER docked pose maintains the B conformation of the pterocarpan ring while the docked pose of the (+)-glyceollin I displays the A conformation, shown in Fig. 5C and D [47]. Furthermore, the (−)-glyceollin I docked pose is stabilized by H-bonds with a H_2O that is H-bonded to Thr 347; whereas the (+)-glyceollin I enantiomer pose maintains the opposite orientation and cannot be stabilized by or interact with H_2O or Thr 347 (Fig. 5E and F). These differences in ER binding conformations and H-bonding patterns may account for the differences observed in ERE transcriptional and gene expression displayed by the glyceollin I enantiomers.

4. Discussion

The unique structural feature of the pterocarpans consists of a tetracyclic system having fused benzofuran–benzopyran rings [48] which also results in two chiral centers in the positions 6a and 11a.

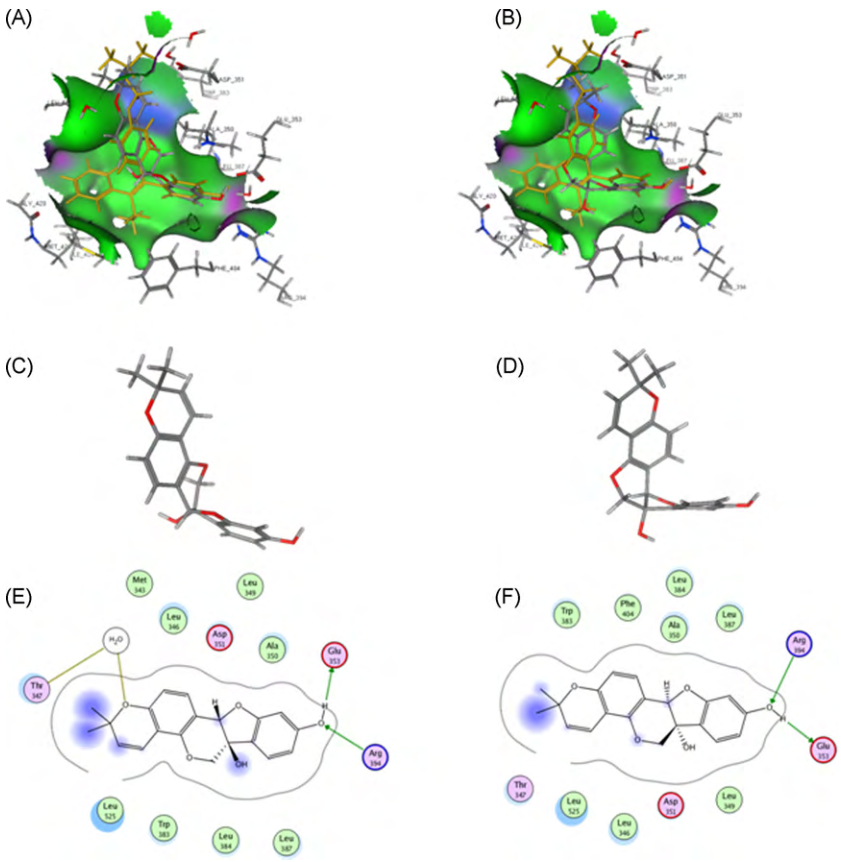

Fig. 5. Molecular modeling of glyceollin I enantiomers. 4-Hydroxytamoxifen and representative docking poses of the (+)-glyceollin and (−)-glyceollin I enantiomers in the binding cavity of the 3ERT structure of ERα. The crystal determined pose of 4-hydroxytamoxifen is shown in yellow for reference and the residues that make up the binding cavity are shown in atom type coloring. These include Aspartic Acid 351 (top right) that is responsible for the ionic interaction with the tertiary amine of 4-hydroxytamoxifen and both Arginine 394 and Glutamate 353 (lower right) that hydrogen bond to the 4-hydroxytamoxifen phenolic ring. Also shown is the receptor binding cavity surface displayed by MOE (Gaussian Contact) where cavity properties are depicted as green for hydrophobic, blue for mild polar and magenta for H-binding. The highest scoring poses of (+)-glyceollin (A) and (−)-glyceollin I (B) enantiomers docked in the 4-OH-tamoxifen binding site of the 3ERT crystal structure. The docked conformations of (+)-glyceollin (C) and (−)-glyceollin I (D) enantiomers. 2-dimensional ligand interaction diagrams that illustrate the different binding modes predicted for the (+)-glyceollin (E) and (−)-glyceollin I (F) enantiomers.

It is well known that only compounds with a *cis* fusion of these "B" and "C" rings are present in nature. Similarly, computational studies have shown that the *trans* isomers are much less energetically favored than the *cis* isomers [27]. The aim of this research is to evaluate the role that pterocarpan phytoalexin glyceollin I enantiomers play in breast cancer treatment and to investigate the potential differential activity of the two glyceollin I enantiomers.

Previously Korach et al. have shown DES metabolites and analogs produced enantiomers exhibited differential binding and biological activity toward the estrogen receptors. The results of these studies suggest that the poor biological activity of one of the metabolites (indenestrol A) may be related to the differential ER interaction of its enantiomers [33,34,37]. Weiser et al. showed that the ERβ agonist, DPN, exists as a racemic mixture of two enantiomers, R-DPN and S-DPN. Through extensive biological assays Weiser et al. found that the S-DPN enantiomer is the biologically active form of DPN [38]. Setchell et al. have shown that equol produces two enantiomers which showed different binding affinities for the estrogen receptors and produced different biological activities [40,41]. These previous studies show that the biological activity of several enantiomers is very different and that only one of the enantiomers may be bioactive.

In this study we have shown that the two glyceollin I enantiomers bind to the estrogen receptors with similar binding affinities; however, the biological potency is not only determined by ligand-binding affinity but requires transcriptional activation of the receptor and target gene expression [49]. Our docking studies show that both glyceollin I enantiomers may bind to the ligand-binding pocket of 3ERT, which is the 4-OH-Tam induced "antagonist" form of the ER, in a similar manner. It should be noted however, that depending on tissue and gene, 4-OH-Tam is a partial agonist or partial antagonist and that the ER binding mode observed in the 3ERT structure is likely induces both cases. In the docked poses of the glyceollin enantiomers, both have their phenolic D-rings interacting with the steroid A-ring site of ER utilized by 4-OH-Tam. At the same time, these docking studies present different ring conformations and different H-bond patterns to Thr 347 between the two glyceollin I enantiomers. The similarities of these two proposed binding modes may account for the similar ER binding affinity observed for both glyceollin I enantiomers. At the same time, the differences in binding modes they also may account for enantiomer selective biological and transcriptional activity presented in this report. For example, the (−)-glyceollin I inhibited the expression of an ERE-luc reporter gene in HEK 293 and MCF-7 cells suggesting that the (−)-glyceollin I may exert antiestrogenic activity, via an ER dependent mechanism. The (−)-glyceollin I enantiomer behaves as an antagonist, while the (+)-glyceollin I enantiomers slightly increased ERE transcriptional activity in MCF-7 and HEK293 cells. These results suggest that the (+)-glyceollin I enantiomer may exhibit slight estrogenic properties. These results show that the antiestrogenic activity previously observed for natural glyceollin I, resides only in the same synthesized enantiomer,

and that these properties are not shared by the optical antipode ((+)-glyceollin I) which was also obtained by chemical synthesis. This is consistent with the modeling and docking analysis of the glyceollin I enantiomers which suggests that the conjugated ring system of the enantiomers extends out of the binding pocket into the same channel used by the aryl amine side chain of 4-OH-Tam to displace helix-12 into the antagonist configuration of ER. Differences in the biological activity between the glyceollin I enantiomers may result from the different ER binding modes suggested by the docking study where the (−)-glyceollin I enantiomer interacts with Thr 347 through a H_2O link; while this interaction is not possible for the (+)-glyceollin I enantiomer. The importance of this interaction for the onset of an estrogenic response may further explain the antiestrogenic activity observed by the (−)-glyceollin I enantiomer. Thus, we suggest that while both enantiomers can take on a conformation that allows binding to the ER, only the (−)-glyceollin I enantiomer, with its H-bond network to Thr 347 will actually promote an antagonist conformation of the receptor similar to that induced when 4-OH-Tam is an antagonist. At the same time, the (+)-glyceollin I enantiomer binding to ER without H-bonds to Thr 347 may be unable to promote the antagonist conformation of the ER and may actually stabilize an agonist ER conformation similar to what may be induced by the 4-OH-Tam in tissues such as the uterus. With our docking studies involving only a fixed receptor conformation, it is not clear how the ER structure would change after the different binding interactions of either glyceollin I enantiomer.

We also demonstrated that the (−)-glyceollin I enantiomer regulates several genes differentially compared to the (+)-glyceollin I enantiomer and E_2 as demonstrated by the gene superarray analysis (Table 1), suggesting an alternant mechanism of action. Herein, we have shown that the (−)-glyceollin I enantiomer down-regulates PgR while the (+)-glyceollin I enantiomer and E_2 up regulate PgR. Additional genes, such as NGFR and GABRP, showed a difference in the biological activity of the glyceollin I enantiomers. The (−)-glyceollin I enantiomer stimulated NGFR, a TNFR superfamily member, 7-folds greater than the (+)-glyceollin I enantiomer and E_2. GABRP, gamma-aminobutyric acid A receptor, was down-regulated by the (−)-glyceollin I enantiomer nearly 3-folds more than the (+)-glyceollin I enantiomer and 2-folds more than E_2. These changes in gene expression have shown that the glyceollin I enantiomers behave biologically different and support the transcriptional activity data shown above. There is a lack of obvious evidence for the relationship between the structure and antiestrogenic activity of pterocarpans. However, computational and experimental studies have shown that the *cis* fused ring of pterocarpans exhibits two conformations free of high energy steric clashes: conformation "A" and "B" [47]. While docking studies suggest similar binding modes for the glyceollin I enantiomers, the (−)-glyceollin I enantiomer takes on the "B" conformation (Fig. 5E) with additional stabilizing H-bond and the (+)-glyceollin I enantiomer maintains the "A" conformation (Fig. 5F) without the benefit of such a stabilizing H-bond. These results may account for the differences in ERE transcriptional activity and gene expression of the glyceollin I enantiomers.

In summary, based upon biological activity, structural characteristics and molecular modeling, the glyceollin I enantiomers behave differently. Several studies have identified enantiomer pairs which have different biological activities [38–41]. Our results indicate that the (−)-glyceollin I enantiomer is an antiestrogen and does not induce estrogen responsive genes via superarray and ERE transcriptional activity. Based upon the structural characteristics and biological activity, the potential use of (−)-glyceollin I enantiomer as a lead compound for the development of novel antiestrogens may prove useful in the treatment of breast carcinoma.

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