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Subglutinol A, an immunosuppressive α-pyrone diterpenoid from *Fusarium subglutinans*, acts as an estrogen receptor antagonist

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ABSTRACT

Subglutinol A is an immunosuppressive α-pyrone diterpenoid isolated from *Fusarium subglutinans* that exhibits osteogenic activity. Several non-steroid mycotoxins isolated from various strains of *Fusarium* fungi exhibit female steroid hormone activities. In this study, we characterized the estrogenic activity of subglutinol A (**1**). Subglutinol A blocked the 17β-estradiol-induced activation of reporter plasmids and endogenous estrogen-responsive target genes in a dose-dependent manner and efficiently destabilized ER proteins as shown using the estrogen receptor antagonist ICI 182,780. Subglutinol A also displaced the specific binding of [^{3}H]17β-estradiol from ER in MCF-7 whole-cell ligand binding assays. These data demonstrate the potential of subglutinol A as an ER antagonist though its competition with 17β-estradiol for direct ER association.

1. Introduction

Subglutinol A (**1**, Fig. 1) is an immunosuppressive α-pyrone diterpenoid that can be isolated from *Fusarium subglutinans*, an endophytic fungus from the vine *Tripterygium wilfordii* Hook. F. (Celastraceae) [1,2]. *T. wilfordii* has long been used as an anti-inflammatory ingredient in traditional Chinese herbal medicines [3,4]. Formulated extracts of *T. wilfordii* are Chinese FDA-approved drugs (Lei Gong Teng tablets, CFDA approval #Z42021534) for rheumatoid arthritis, psoriasis, lupus-associated autoimmune nephrotic syndrome, and autoimmune hepatitis. Subglutinol A has drawn a great deal of attention as a promising new immunosuppressive agent due to its potency and lack of toxicity [1,2]. After accomplishing the first total synthesis of subglutinol A [5,6], the efficacy of subglutinol A was demonstrated in eliminating Th1 and Th17 responses *in vitro* and in suppressing inflammation *in vivo* [5–7]. Preliminary studies also suggest that subglutinol A might regulate immune responses through a particularly unique mechanism. These results support the expected potential of subglutinol A as a new immunosuppressive agent.

Mycoestrogens such as zearalenone are non-steroidal estrogens synthesized by several *Fusarium* fungi [8,9]. These compounds act as weak estrogens, share structural similarities with 17β-estradiol (E2), and exert similar molecular effects by competitively binding estrogen receptors (ERs), thereby leading to disruption of receptor signaling and hormone production [10]. Mycoestrogens and plant-derived phytoestrogens have shown clinical potential for the relief of menopausal symptoms and chronic diseases such as osteoporosis and atherosclerosis [11,12]. Both the harmful and beneficial estrogenic effects of phytoestrogens on human and animal health, combined with a newfound public interest in hormone replacement therapy, have accelerated the search for estrogen mimics from various sources [13]. Selective estrogen receptor modulators (SERMs) are chemically diverse, non-steroidal compounds that interact with ERs through various estrogen agonist and antagonist activities in different tissues [14]. The main clinical utilities of SERMS are in the prevention and treatment of breast cancer and in the protective maintenance of bone and serum lipid profiles in postmenopausal women [15] For example, raloxifene, a benzothiophene SERM, is approved for the prevention and treatment of postmenopausal osteoporosis and for the reduction of invasive

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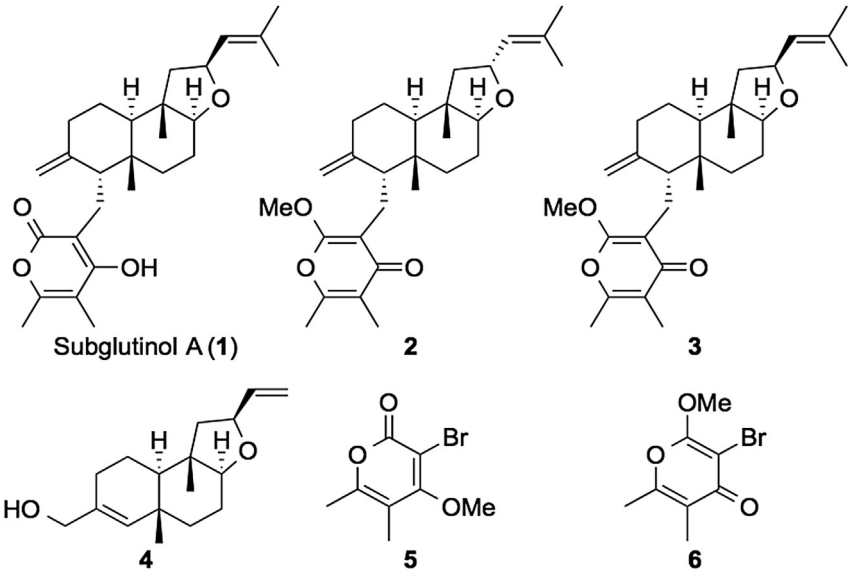

Fig. 1. Structure of subglutinols.

breast cancer risk [16]. A recent study showed that raloxifene provides neuroprotection by alleviating Th17 cell infiltration in the CNS of experimental autoimmune encephalopathy in an ovariectomized animal model of multiple sclerosis [17].

The structural resemblance to steroid hormones and the immunosuppressive effect of subglutinol A (**1**) prompted us to examine its estrogenic activity. The evidence is provided herein that subglutinol A (**1**) acts as an antiestrogen compound in MCF-7 breast cancer cells. These results demonstrate the promising potential of subglutinol A (**1**) as a new SERM candidate with antiestrogenic activity in addition to its previously reported osteogenic and anti-inflammatory effects.

2. Materials and methods

2.1. Materials

The percentage purity of subglutinol A (**1**) used for the present study was above 95% as determined by ^{1}H NMR. E2 was purchased from Sigma (St. Louis, MO, USA) and dissolved in 100% ethanol. ICI was obtained from Tocris (Bristol, UK). The ERE-tk81-luc, constructed by inserting the fragment of the herpes simplex thymidine kinase promoter and two copies of the vitellogenin ERE into pA3luc (ERE-luc), was a kind gift from Dr. Larry Jameson. Expression vectors for ERα (HEGO) were received from Dr. Pierre Chambon.

2.2. Cell culture and treatment

MCF-7 cells were grown at 37 °C in a humidified atmosphere of 95% air/5% CO_2 in phenol red-free RPMI supplemented with 10% fetal bovine serum (FBS; WelGENE, Seoul, Korea). Before treatment, the cells were washed with phosphate-buffered saline (PBS) and cultured in RPMI/5% charcoal-dextran stripped FBS (ST-FBS) for 1 day to eliminate any estrogenic source before treatment. All of the compounds were added to the medium such that the total solvent concentration was lower than 0.1%. An untreated group served as a control.

2.3. Transient transfection and luciferase assay

MCF-7 cells were seeded in 24-well plates at a density of 5×10^4 cells/well. After 24 h, plasmids were transiently transfected with ERE-luciferase reporter plasmids into the cell by using the polyethylenimine (PEI; Polysciences, Warrington, PA, USA) method. Further cells were incubated for 12 h and later treated with E2 and/or ICI. After 24 h of treatment, plates were frozen at −70 °C at least for 2 h to lyse the cells. Luciferase activity was determined with an AutoLumat LB953 luminometer (Lumat LB 9507, EG & G Berthold, Bad Widbad, Germany) using the luciferase assay system (Promega Corp., Madison, WI, USA) and expressed as relative light units. All transfection experiments were repeated at least three times.

2.4. Reverse transcription (RT)–Polymerase chain reaction (PCR)

Total RNA was extracted using Trizol Reagent according to the manufacturer's instruction. RNA pellets were dissolved in diethylpyrocarbonate-treated water. The yield of RNA was quantified by spectroscopy at 260 nm. Samples were aliquoted and stored at −80 °C until further processing. To synthesize first strand cDNA, 3 μg total RNA was incubated at 70 °C for 5 min with 0.5 μg of random hexamer and deionized water (up to 11 μl). The reverse transcription reaction was performed using 40 units of M-MLV reverse transcriptase (Promega, Madison, WI, USA) in 5 × reaction buffer (250 mmol/l Tris–HCl; pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), RNase inhibitor at 1 unit/μl, and 1 mM dNTP mixtures at 37 °C for 60 min. Quantitative real-time PCR (qPCR) was performed using iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The primers used were: β-actin sense primer, 5′-CAAATGCTTCTAGGCGGACTATG-3′; β-actin anti-sense primer, 5′-TGCGCAAGTTAGGTTTTGTCA-3′; GREB1 sense primer, 5′-AAATCGAGGATGTGGAGTG-3′; GREB1 anti-sense primer, 5′- TCTCACCAAGCAGGAGGAG-3'; PR sense primer, 5′-CGCGCTCTACCCTGCACTC-3′; PR anti-sense primer, 5′-TGAATCCGGCCTCAGGTAGTT-3′. A final volume was 25 μl, and an iCycleriQ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used for qPCR. The amplification data were analyzed by iQ™ 5 optical system software version 2.1 and calculated using the $\Delta\Delta C_T$ method. The $\Delta\Delta C_T$ method was used to calculate relative mRNA expression.

2.5. Western blot analysis

Protein was isolated in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS)

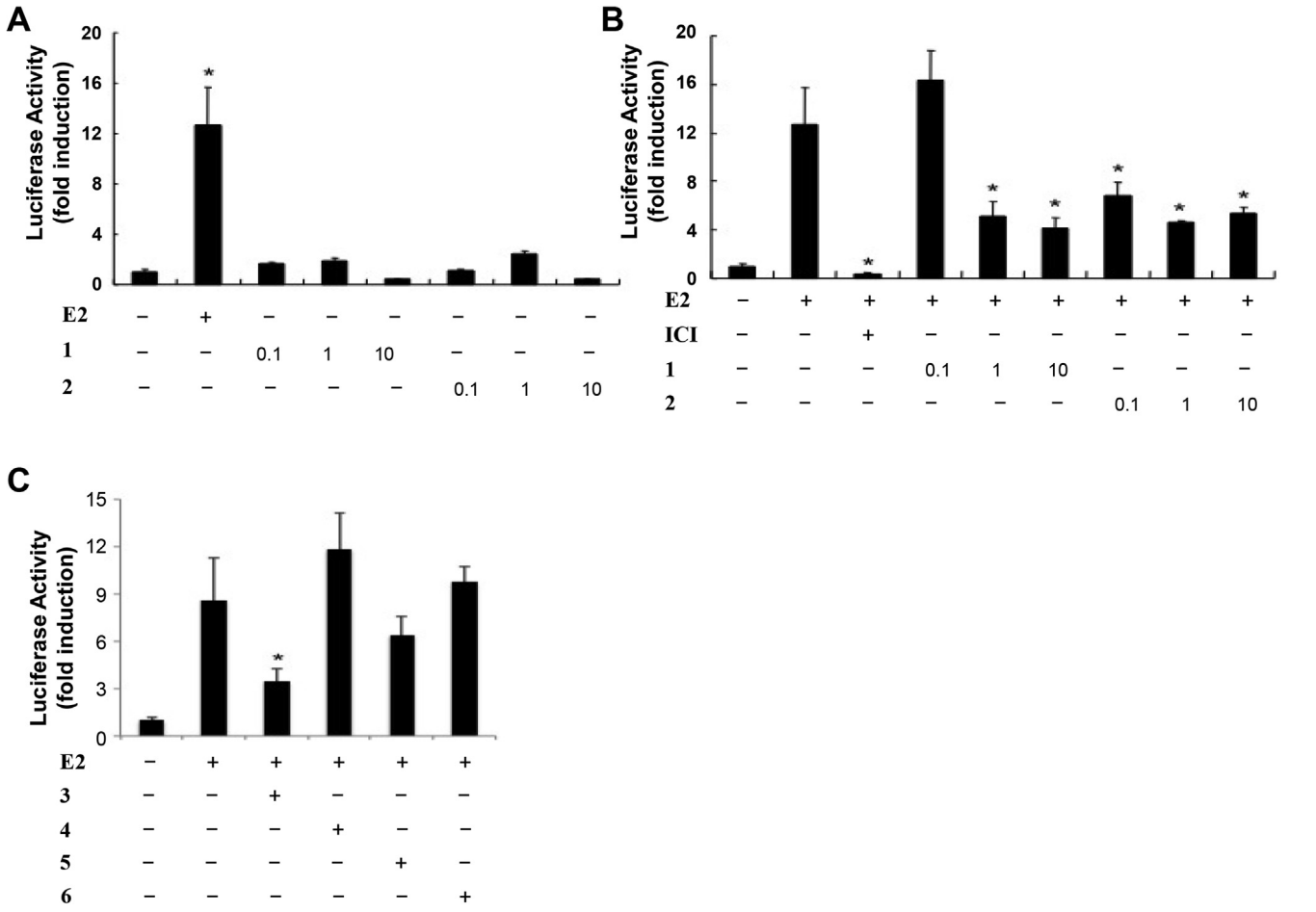

Fig. 2. Effects of subglutinols on ERE-luciferase reporter activity. A, B) MCF-7 cells were treated with 0.1–10 μM of subglutinol A (**1**) or **2** with or without 10 nM E2 as indicated for 24 h followed by transient transfection. ICI was used to block the effect of E2 as a control. Cell lysates were prepared and analyzed by luciferase assays. C) MCF-7 cells were transfected with ERE-Luc and treated with subglutinols 3–6 with 10 nM E2 as indicated. After treatment, luciferase expression was determined. The data are representative of at least three independent experiments performed in triplicate and presented as relative luciferase activity. $^{*}p < 0.05$.

with protease inhibitor cocktail (Sigma, St. Louis, MO, USA) on ice for 1 h and then centrifuged for 20 min at 13,000× *g*. Supernatant was collected and protein concentrations were measured using the Bradford method (Bio-Rad, Hercules, CA, USA). Proteins were dissolved in sample buffer and boiled for 5 min prior to loading onto an acrylamide gel. After SDS-PAGE, proteins were transferred to a polyvinyl denedifluoride membrane, blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 60 min at room temperature. The membranes were incubated for 2 h at room temperature with antibody. Equal lane loading was assessed using β-actin monoclonal antibody (Sigma, St. Louis, MO, USA). After washing with TBST, blots were incubated with 1:5000 dilution of the horseradish peroxidase conjugated-secondary antibody (Invitrogen, Grand Island, NY, USA), and washed again three times with TBST. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA). ERα was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). (San Jose, CA, USA).

2.6. ER binding assay

MCF-7 cells were stripped of any estrogen by keeping them in phenol red-free DMEM supplemented with 10% CD-FBS for 2 days. Cells were incubated with 1 nM [2,4,6,7-^{3}H]E2 (89 Ci/mmol; PerkinElmer, Waltham, MA, USA) and a 100-fold excess of nonlabeled E2 (100 nM) or 1–10 μM subglutinols for 1 h at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Aliquots of the medium were measured before and after the incubation with the cells to determine the total uptake of E2 into the cells. After removal of the medium, cells were washed with ice-cold PBS/0.1% methylcellulose twice, harvested by scraping and centrifugation, and lysed with 100% ethanol 500 μl per 60 mm dish for 10 min at room temperature. The radioactivity of extracts was measured by liquid scintillation counting. The specific binding (%) to each ER was determined as follows: $[(dpm_{sample} - dpm_{nonspecific})/(dpm_{veh} - dpm_{nonspecific})] \times 100$.

2.7. Statistical analysis

Data were expressed as means ± SD, and statistical analysis for single comparison was performed using the Student's *t* test. The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Inhibition of ER-mediated transcriptional activation in MCF-7 cells

Proper ligand binding to ERs initiates transcriptional activation through specific estrogen response elements (EREs) in target genes

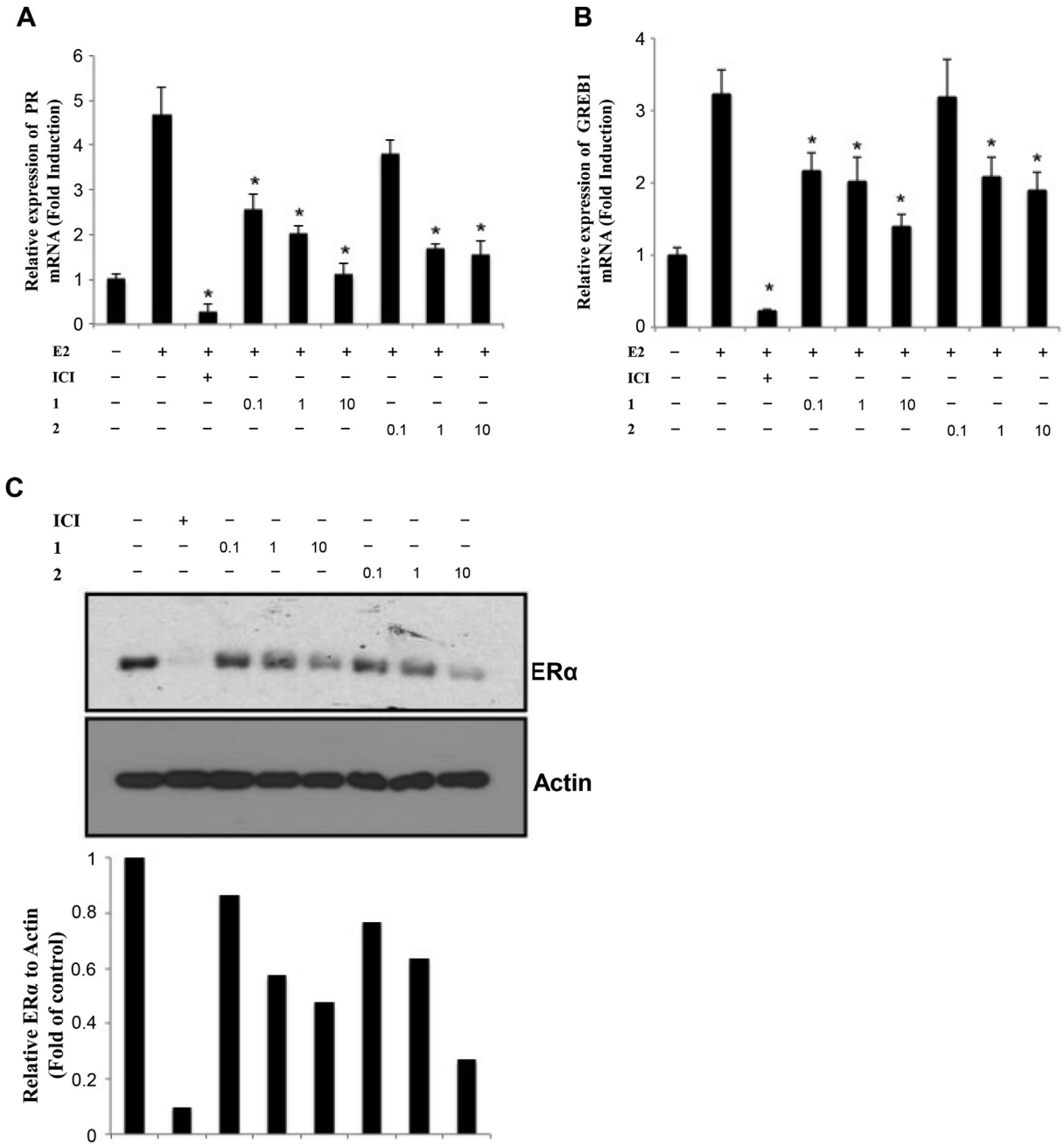

Fig. 3. Effects of subglutinol A (**1**) and **2** on endogenous estrogen responsive PR and GREB1 genes and ERα protein levels. MCF-7 cells were treated with E2 10 nM or subglutinol 0.1–10 μM for 24 h. An untreated group served as a control. The qRT-PCR results for PR (A), GREB1 (B) in MCF-7 cells are shown ($n = 3$). *$p < 0.05$. After the incubation, the cells were lysed and total protein extracts were resolved by SDS-PAGE and immunoblotted using an anti-ERα antibody or an anti-β-actin antibody (C). ER densitometry values are expressed as a percentage of the control (down). These experiments were repeated at least three times.

[18]. To examine the estrogenic activity of subglutinols, the estrogen hormone activity of subglutinol A (**1**) and several structurally related analogs **2**–**6** (Fig. 2) were investigated by examining the activation of reporter plasmids containing ERE transiently transfected intoMCF-7 cells. E2, which was used as a positive control, significantly increased ERE-luciferase activity after 24 h of treatment at a concentration of 10 nM (Fig. 2A). However, neither **1** nor **2** enhanced luciferase activity at concentrations from 0.1 to 10 μM after the same treatment duration (Fig. 2A). In assessing these results, it was noticed that the reporter activity of 10 μM subglutinol was significantly lower (Fig. 2A) than that of the untreated control. This may be the result of blocking trace amounts of residual estrogen present in FBS in the media. To explore the possibility of subglutinol acting as an antiestrogen, its effect on ERE-reporter activity was examined with concomitant treatment with 10 nM E2. ICI 182,780 (ICI), a pure antiestrogen, completely blocked E2 activity at a concentration of 1 μM, which was sufficient to saturate almost all of the ERs in the cells. Although not as effective as ICI, subglutinols blocked E2-induced luciferase activity at concentrations of 1–10 μM (Fig. 2B).

To identify the structural elements of subglutinol A (**1**) required for inhibitory activity on ER-mediated transcription, several structural derivatives were evaluated (Fig. 2C). ERE-luciferase reporter assays were used to assess the antiestrogenic activity of subglutinol

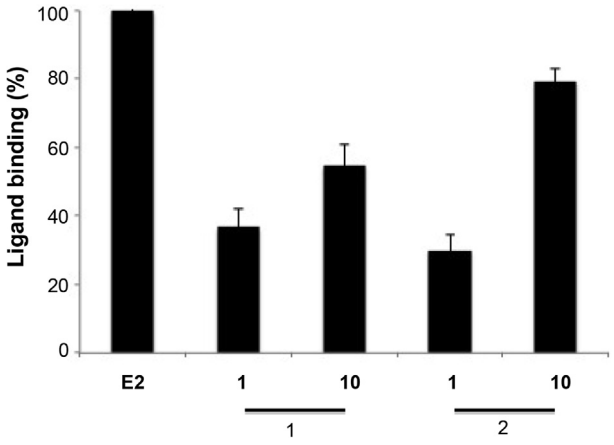

Fig. 4. Competitive binding of subglutinol A (**1**) and **2** to ER in MCF-7 cells. Cells were incubated with 1 nM ^{3}H-labeled E2 (89 Ci/mmol) plus vehicle, nonlabeled E2 (100 nM), or subglutinol (as indicated) for 1 h. Radioactivity of ethanol extracts of cell lysates was measured by scintillation counting. Specific binding was calculated as total binding minus nonspecific binding. Data were expressed as percentage of ligand binding, which is (total binding − nonspecific binding)/total binding × 100.

derivatives **3**–**6**. Compound **3** exhibited a useful level of antiestrogenic activity, while **4**–**6** were completely inactive. Initial structure-activity relationship studies suggested that the α- or γ-pyrone moiety plays an important role in antiestrogenic activity by acting as an electrophile to form a covalent link to amino acid residues, e.g., cysteine, in the active site of molecular target(s) [19].

To further evaluate the potential of subglutinols as repressors of ERs, their effects on GREB1 and Progesterone receptor (PR) were examined, two well-studied estrogen-responsive genes in MCF-7 cells. qRT-PCR assays showed that the subglutinols blocked the transcription of E2-induced GREB1 and PR in a dose-dependent manner after 24 h treatments (Fig. 3A, B). Ten micromolar subglutinol suppressed E2-induced PR and GREB1 gene expression to the control level. These data indicate that subglutinols act as weak ER antagonists that disrupt ER-mediated pathways.

3.2. Downregulation of ERα protein levels in MCF-7 cells

ICI, also known as faslodex, is a selective estrogen receptor downregulator that dramatically downregulates ERs to the point of completely blocking their activity on gene transcription with no mixed agonist activity [20]. The effect of subglutinols on the ERα protein was evaluated to determine the mechanism of subglutinol-induced repression of ERα transcriptional activity. ERα protein levels were decreased by treatments with **1** and **2** in a dose-dependent manner relative to the vehicle control (Fig. 3C). The effects of **1** and **2** were less prominent than that of 1 μM ICI, implying that their efficacy is not as high as that of ICI. The reported affinity of ICI is approximately 0.1 nM. These data indicate that one possible mechanism of ERα transcriptional repression by subglutinol A (**1**) is the degradation of ERα proteins. This mechanism would confirm that subglutinols act as weak ER antagonists.

3.3. Subglutinols compete with estrogen binding to ERs

The activation of ERs is initiated by ligand binding and subsequent binding to DNA. To determine whether the observed effects of subglutinols occur via direct binding to ERs, the ability of **1** and **2** was examined to compete with [^{3}H]-labeled-E2 for ER binding in MCF-7 cells (Fig. 4). Specific binding was calculated as the total binding minus the amount of nonspecific binding (determined in the presence of a 100-fold excess of unlabeled E2 or subglutinol at the concentrations indicated in Fig. 4). A competitive binding assay showed that compounds **1** and **2** displaced [^{3}H] E2 in a dose-dependent manner (Fig. 4). Compounds **1** and **2** displaced approximately 46% of [^{3}H]-labeled E2 binding to ERs at a concentration of 10 μM. These data indicate that subglutinols act as dose-dependent antagonists, inhibiting the transcriptional activation of ERs with an estimated affinity of 10 μM.

4. Discussion

This study evaluated the estrogen-like activity of subglutinol A (**1**), a natural product isolated from *F. subglutinans*. Subglutinol A (**1**) effectively blocks the transcriptional activation of ERα and induces ERα degradation in a dose-dependent manner. Binding of E2 to ERα was competitively inhibited in the presence of subglutinol A (**1**). To the best of our knowledge, this is the first report of the antiestrogenic activity of subglutinol A(**1**). Many estrogenic compounds that have been identified in food, including herbs, grains, vegetables, and fruits, fall into one of three major classes: isoflavones, lignans, and coumestans [11] However, natural antiestrogens are not as abundant as phytoestrogens. Among the few natural antiestrogenic compounds, indole-3-carbinol (I3C) is a well-studied antiestrogenic phytochemical derived from the hydrolysis of glucobrassicin produced in *Brassica* cruciferous vegetables, including cabbage, broccoli, and brussel sprouts [21]. I3C ablates ERα expression and the estrogen-dependent proliferation of estrogen-responsive human breast cancer cells [22]. I3C also exhibits potent anticarcinogenic properties against a wide range of human cancers [23]. One option for the clinical management of hormone-dependent breast cancers is the use of SERMs such as tamoxifen that can block ligand-dependent receptor activation [24]. Further in vivo animal studies are necessary to demonstrate subglutinol A (**1**) as a new SERM candidate and antiestrogen therapeutic agent for estrogen-sensitive breast cancers.

Conflict of interest

None.

Acknowledgments

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Transparency document

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