藥學碩士學位論文

Semi-synthesized diosgenin glycosides inhibit cell growth through estrogen receptor signaling pathway and induce apoptosis in MCF-7 breast adenocarcinoma

디오스게닌 유도체에 의한 MCF-7 유방암 세포의 항암활성연구

2012년 8월

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약학과 천연물과학전공

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이 논문을 한리나 석사학위논문으로 제출함

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한리나

한리나의 석사학위논문을 인준함

2012년 8월

위 원 장:_____ (인)

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위　　원:_____ (인)

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논문제목 : Semi-synthesized derivatives of diosgenin glycosides inhibit cell growth through estrogen receptor signaling pathway and induce apoptosis in MCF-7 breast adenocarcinoma

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ABSTRACT

Semi-synthesized diosgenin glycosides inhibit cell growth through estrogen receptor signaling pathway and induce apoptosis in MCF-7 breast adenocarcinoma

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Estrogen receptor, overexpressed in 70% breast cancer, is a potential interest for several disease indications (*e.g.* breast cancer, metabolic diseases, or osteoporosis) and an important therapeutic target in the clinical treatment of breast cancers. In order to synthesize the potential down-regulator of estrogen receptor, derivatives of diosgenin glycosides (abbreviated as DG) were designed by conjugation of diosgenin and disaccharide structure (α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranose) from β-hederin. Each of thirteen DG compounds (00 to 12) bears an exclusive structure according to the glycosylated sugar groups

on disaccharide structure. As a result, DG series compounds showed great differences in cytotoxicity and ER inhibition effect. Among the six cytotoxic DG compounds, DG03 showed the most potential activity as an ER down-regulator and inhibited cell growth by inducing apoptosis. Experimental data showed that DG03 specifically suppressed the protein and mRNA expression of ERα in MCF-7 cells. ER-ERE (estrogen responsive element) binding activity was tested by electrophoretic mobility shift assay (EMSA), and both classical and non-classical DNA binding activities were decreased by treatment of DG03. Therefore, ER-mediated gene expression, such as c-Myc, cyclinD1 and pS2 were also reduced, indicating the possibility of cell cycle arrest and inactivation ER signaling. Further research also demonstrated that DG03 reduced phosphorylation of tyrosine kinase Src and inhibited signal transduction through phosphoinositide 3-kinase (PI3K/AKT) and MAPK pathways. In order to examine the apoptotic effect, three kinds of breast cell lines, such as non-tumorigenic epithelial MCF-10A, ERα-positive MCF-7 and ERα-negative MDA-MB-231 cells were adopted to test apoptotic effect by using fluorescence-activated cell sorting (FACS). As a result, MCF-7 cells showed the most significant apoptosis compared with other two cell lines, indicating that DG03 selectively induced apoptosis in ER-positive MCF-7 breast adenocarcinoma. Overall, ER-targeted semi-synthesized DG03 showed great effectiveness as an ER down-regulator and a potential anti-cancer candidate in the treatment of ER-positive breast cancers.

Key words: Estrogen receptor (ER); Apoptosis; MCF-7 human breast adenocarcinoma, ER signaling pathway, Akt pathway.

Student number: 2010-24171

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I. INTRODUCTION

1.1. Estrogen receptor and breast cancer

Estrogen receptor, a potential interest for several disease indications (*e.g.* breast cancer, metabolic diseases, or osteoporosis), is a member of the nuclear hormone receptor (NR) superfamily that functions as a transcription factor. Estrogen receptors are overexpressed in 70% breast cancer cells, serve as a critical regulatory gene for tumor progression and became an important therapeutic target. There are two isoforms encoded by two independent genes, usually referred to as ERα and ERβ, of which ERα is more predominant in cancer research, while the role of ERβ is still under investigation [1].

ER regulation includes ligand-mediated activation (*e.g.* estrogen) and association with cell membrane proteins, such as epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) (Figure 1) [2, 3]. When activated by estrogen, ER goes through dimerization and translocate to nucleus to regulate gene expressions [4]. Signals produced from receptor tyrosine kinases are sent to nucleus via mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K/AKT) pathways [5]. In transcription level, there are classical and non-classical ER signaling pathways [6]. In the classical transcription pathway, estrogen activated ER binds directly to estrogen responsive element (ERE) and initiate gene expression (*e.g.* pS2), while in the non-classical transcription pathway, ER binds indirectly to non-ERE sites or promoter regions of some genes (*e.g.* cyclin D1, c-myc) by interaction with other transcription factors (*e.g.* AP-1, SP-1). Hence, ERα involvement of cell cycle has been reported (Figure 2) [7-10]. Co-activators, such as SRC-1, SRC-3/AlB1 and IKKα are also required to ER-mediated gene expression [11].

Endocrine therapy of ER over-expressed breast cancer can be mainly classified into three categories, such as selective estrogen receptor modulators (SERMs, *e.g.* tamoxifen), selective estrogen receptor down-regulators (ERDs, *e.g.* ICI 182,780) and aromatase inhibitors (AIs, *e.g.* anastrozole), among which tamoxifen has become a routine therapeutic approach in the US (Figure 3). Moreover, research on ER-targeted drug discovery is still in progress, including natural and synthesized compounds. However, resistance cases have been reported based on experimental and clinical data and further study focused on new drug discovery and novel therapeutic strategies remain as hard problem to solve [12].

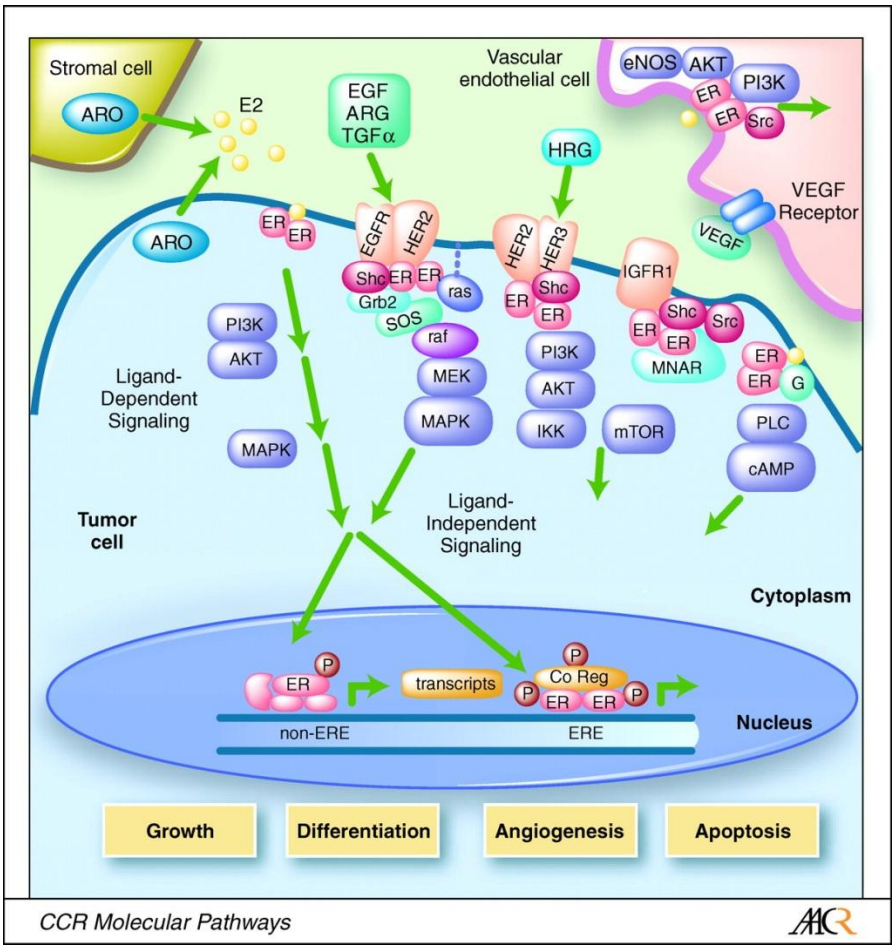


Figure 1. Simplified model of ligand-induced activation and inhibition of ER [2]

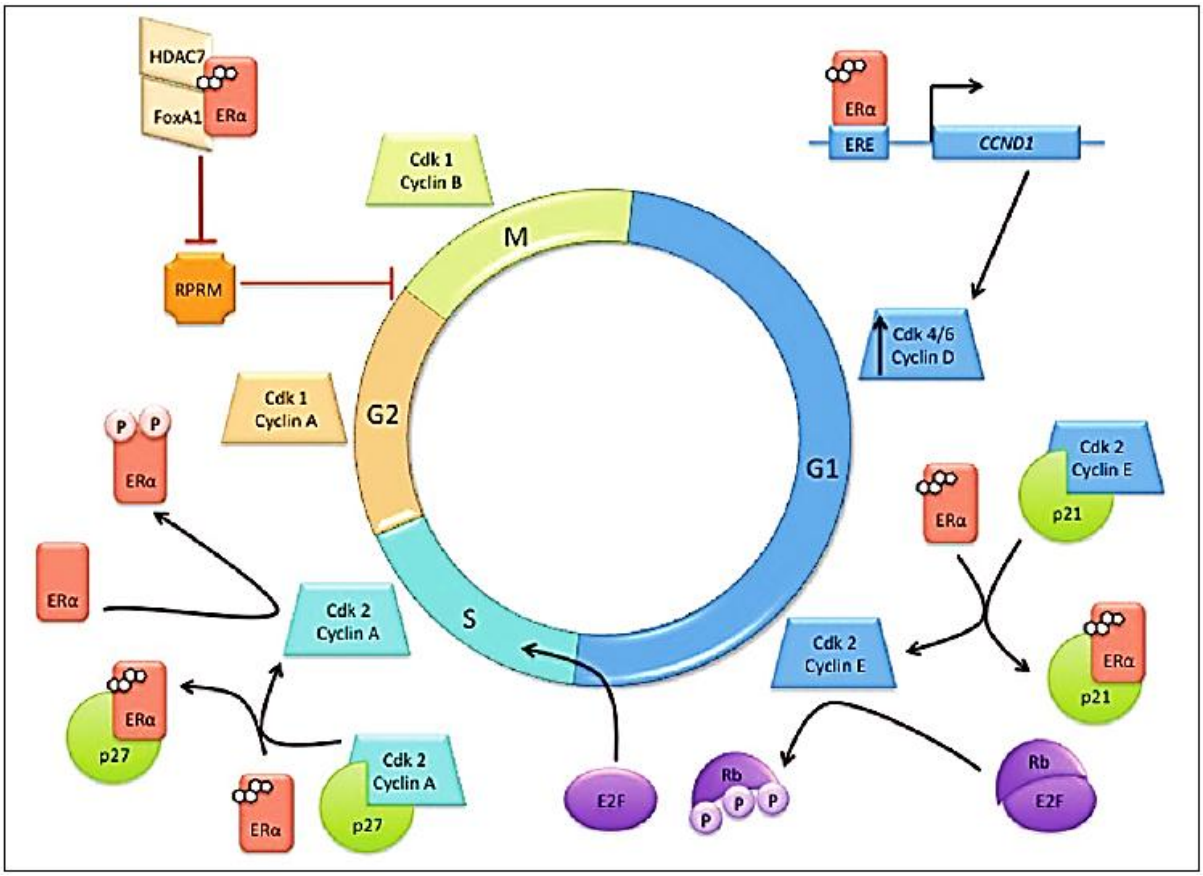
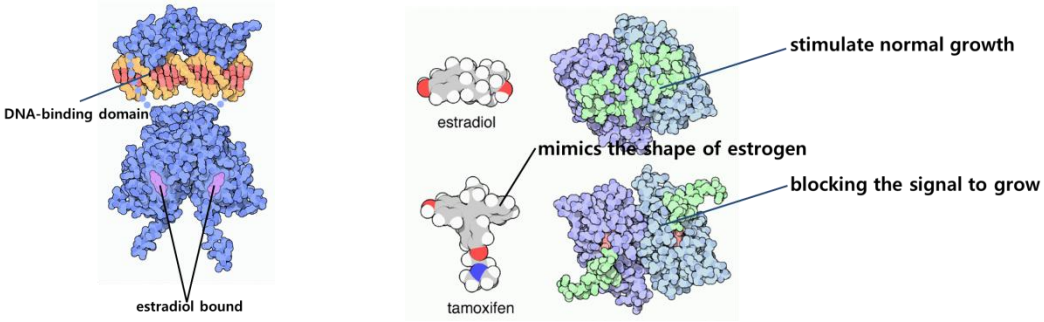


Figure 2. Involvement of ERα in the cell cycle [13]

ERα plays many roles across the cell cycle phases, interacting with cell cycle machinery such as cyclins, cyclin-dependent kinases (cdk), cdk inhibitors, and the retinoblastoma protein (Rb).

(A)



(B)

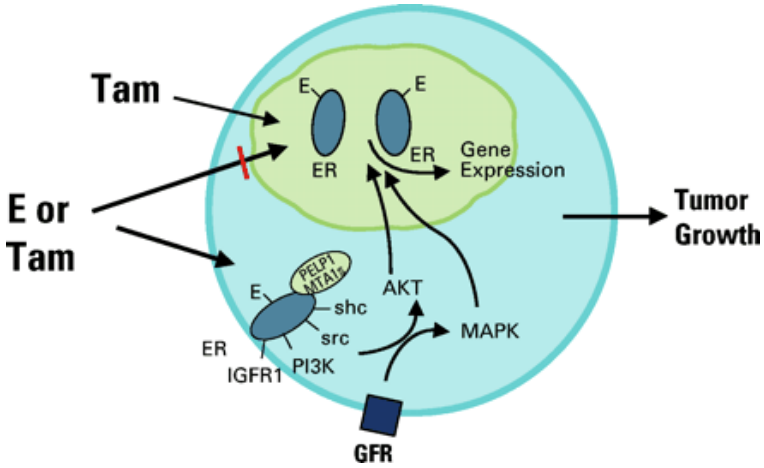


Figure 3. Estrogen receptor and Tamoxifen [14]

(A) ERα is consist of DNA-binding domain and estradiol binding domain. ERα binds with estradiol to stimulate cell growth, while binds with tamoxifen to inhibit normal biological function of ERα. **(B)** Tamoxifen functions as an antagonist of estradiol to suppress tumor growth in ER-positive breast cancers. (**E**: Estradiol, **Tam**: Tamoxifen)

1.2. Apoptosis

Apoptosis is a highly regulated, energy-dependent suicide program, whereby the cell activates a signaling cascade that leads to cell death. The process of this programmed cell death (PCD) is defined by morphological features, such as cell shrinkage, violent blebbing of the plasma membrane and chromatin condensation. The pieces of the cell which remain when the program is complete are termed apoptotic bodies (Figure 4). In tumors, apoptosis is an ideal mode of death adopted by tumor cells and dose not induce an inflammatory response compared to necrosis [15, 16]. Apoptosis is also an important therapeutic target in breast cancers. High levels of apoptosis in breast tumor itself seem to predict worse survival, and sex steroid receptor-negative tumors have greater Apoptotic Index (AIs) than sex steroid receptor-positive ones [17].

The two distinct pathways of apoptosis are extrinsic pathway (Fas and other TNFR superfamily members and ligands) and intrinsic pathway (mitochondria-associated) (Figure 5) [18]. The extrinsic pathway is triggered when death ligands, such as Fas ligand or TNF-α, bind to their cognate death receptors at the plasma membrane, leading to recruitment of specific adaptor proteins, such as Fas-associated death domain and procaspase-8, into a death-inducing signaling complex (DISC). This leads to activation of initiator capase-8 that subsequently activates effector caspases-3 [19]. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily (*e.g.* Fas, TNFR1) that contain a homologous cytoplasmic sequence termed the “death domain” [20]. Adaptor molecules, such as FADD, TRADD, contain death domains so that they can interact with the death receptors and transmit the apoptotic signal to the death-machinery [21]. In contrast, the intrinsic pathway is

characterized by permeabilization of the mitochondria and release of cytochrome c into the cytoplasm. In this process, mitochondria play a central role in the integration and execution of a wide variety of apoptotic signals. Cytochrome c is released in respond to some pro-apoptotic stimuli, such as DNA damage, and activates caspase-9 that further activates effector proteins caspase-3 and caspase-7, which are responsible for destroying the cell from within [22]. The extrinsic and intrinsic pathways converge at the level of the effector caspases, though they were activated by different initiator caspases [19].

Some pro- and anti-apoptotic proteins are involved in the apoptotic pathways and control the balance of the process. The most predominant proteins are the Bcl-2 family of proteins and tumor suppressor protein p53. The anti-apoptotic Bcl-2 proteins, overexpressed in many cancers, play a vital role in the intrinsic apoptotic pathway. The Bcl-2 family includes pro-apoptotic (e.g. Bax, Bak, Bad, Bid, Bim) and anti-apoptotic (e.g. Bcl-2, Bcl-xL, Bcl-w, Mcl-1) proteins, and the interactions of these anti- and pro- apoptotic protein result in the activation of downstream signals, moreover, regulate various apoptotic stimuli [19]. Hence, drugs targeting anti-apoptotic Bcl-2 family proteins may be promising in cancer therapy. Another controversial protein in the apoptotic pathway is p53, which plays a central role in controlling cell cycle progression and apoptosis. The pro-apoptotic activity of p53 has been linked to its transactivation capabilities through several approaches, among which the most intuitive link between p53-mediated transactivation and apoptosis comes from its ability to control transcription of pro-apoptotic members of the Bcl-2 family [23].

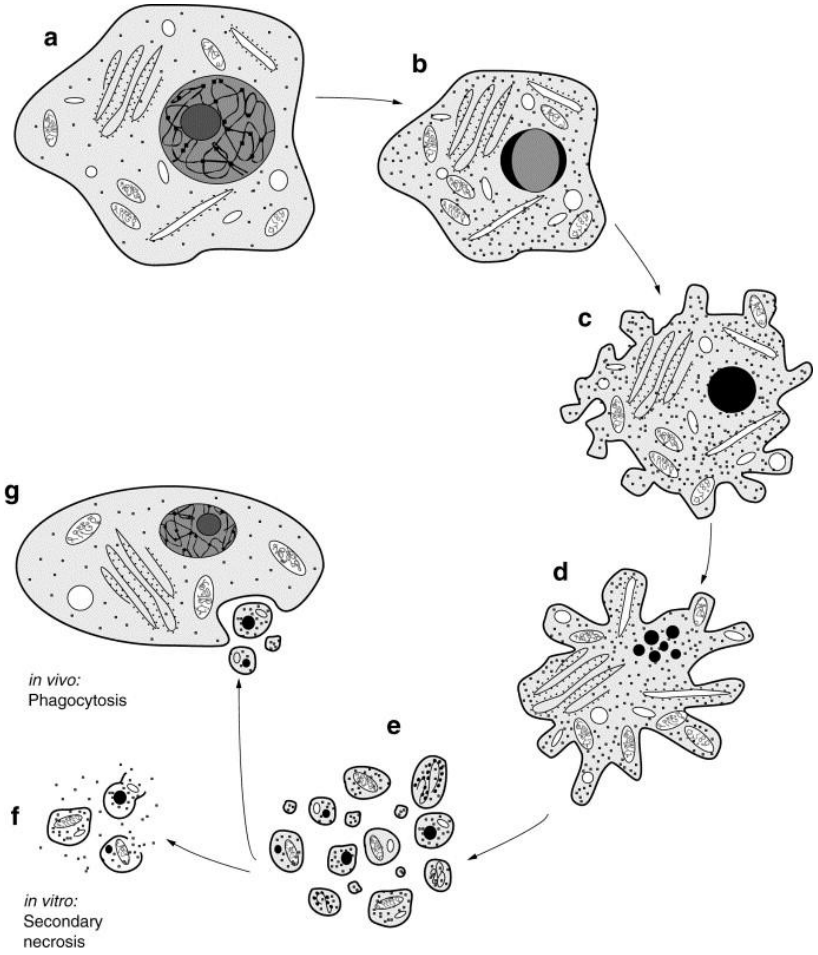


Figure 4. Morphological change of apoptosis [24]

The morphological changes occur during apoptosis. First, the normal cell (**a**) shrinks and the condensed chromatin collapses into crescents around the nuclear envelope (**b**). The membrane then begins to bulge and bleb (**c**), while the nucleus ultimately collapses (**d**). The blebbing increases and the cell finally breaks apart into a number of apoptotic bodies (**e**), which lyse *in vitro* (**f**) and are phagocytosed *in vivo* (**g**).

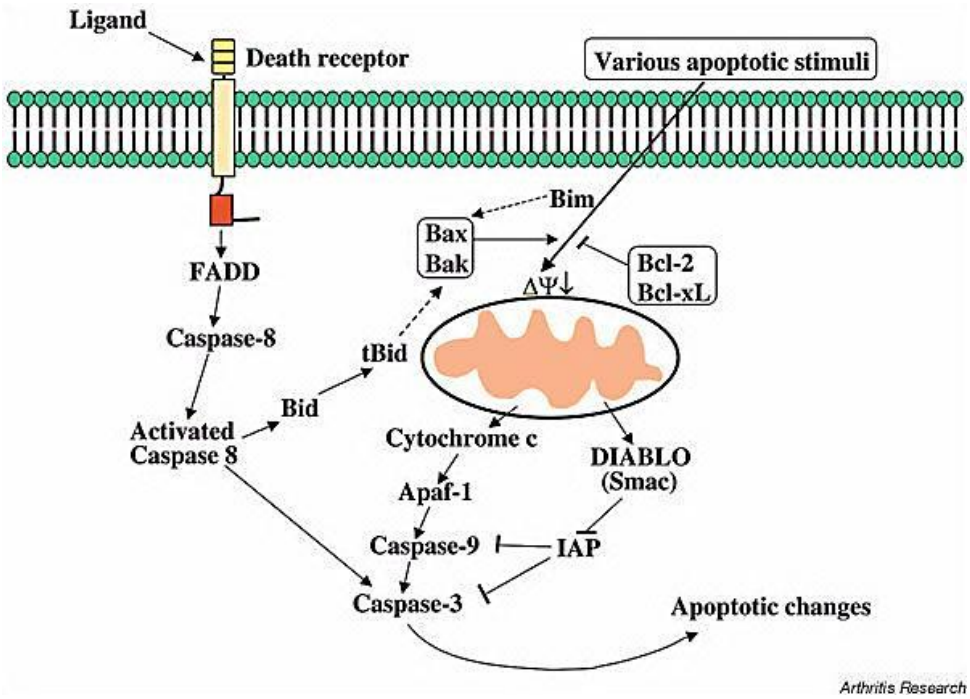


Figure 5. Intrinsic and extrinsic pathway of apoptosis [18]

1.3. Semi-synthesized steroid saponins

Saponins have attracted much attention in recent years due to their various biologically active properties, especially their antitumor activity [25, 26]. Chemically, saponins are composed of a sugar moiety and the aglycone, either a steroid or a triterpenoid, to form steroidal saponins or triterpenoidal saponins, respectively.

Thirteen diosgenyl saponins bearing a unique disaccharide from the natural product β-hederin were synthesized (Figure 6) and their cytotoxicity against human breast cancer cell lines was determined. Six of thirteen compounds showed strong cytotoxic activity, and three of them showed significant inhibition effect of estrogen receptor (DG01, DG03 and DG06) (Table 1). The mechanism study was mainly conducted by using the most potential compound DG03, namely diosgenyl α-L-rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→4)]-α-L-arabinopyranoside. It was indicated that DG series semi-synthesized compounds may function as down-regulators of estrogen receptor alpha in ER-positive MCF-7 breast cancer cells and induce apoptosis via estrogen receptor related signaling pathway.

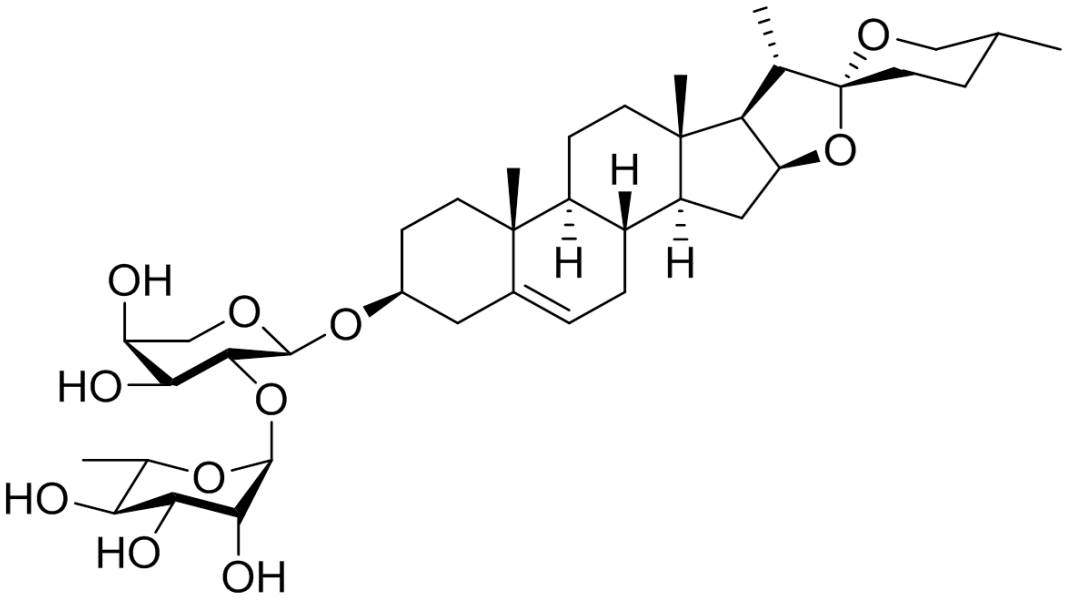


Figure 6. Conjugation of diosgenin and disaccharide structure from β-hederin (DG00)

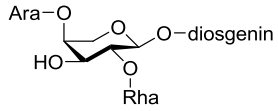
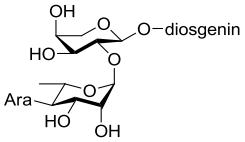
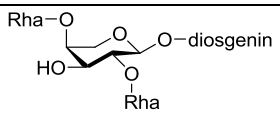
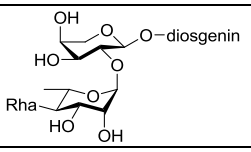
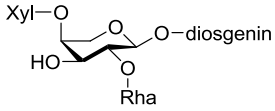
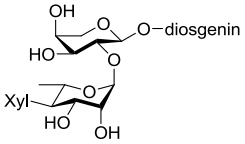
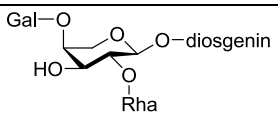
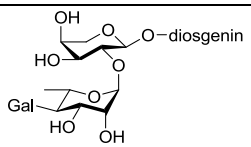
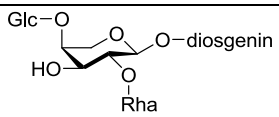
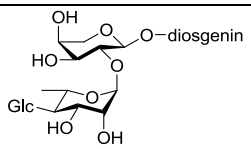
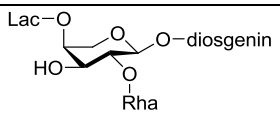
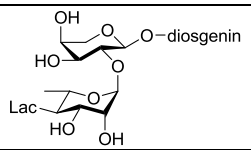
DG01		**DG07**	
DG02		**DG08**	
DG03		**DG09**	
DG04		**DG10**	
DG05		**DG11**	
DG06		**DG12**	

Table 1. Structure of DG series compounds (DG01-DG12)

DG01: Diosgenyl α-L-rhamnopyranosyl-(1→2)-[α-L-arabinopyranosyl-(1→4)]-α-L-arabinopyranoside

DG02: Diosgenyl α-L-rhamnopyranosyl-(1→2)-[-α-L-rhamnopyranosyl-(1→4)]-α-L-arabinopyranoside

DG03: Diosgenyl α-L-rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→4)]-α-L-arabinopyranoside

DG04: Diosgenyl α-L-rhamnopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→4)]-α-L-arabinopyranoside

DG05: Diosgenyl α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside

DG06: Diosgenyl α-L-rhamnopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside

DG07: Diosgenyl α-L-arabinopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside

DG08: Diosgenyl α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside

DG09: Diosgenyl β-D-xylopyranosyl-(1→4) -α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside

DG10: Diosgenyl β-D-galactopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside

DG11: Diosgenyl β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside

DG12: Diosgenyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside

1.4. Purpose of the study

The study is aimed at *in vivo* mechanism study of semi-synthesized DG compounds and evaluation of the value of DG series compounds as a candidate for anti-cancer agent.

Though it has shown cytotoxic activity in several cancer cell lines, such as A549 human lung adenocarcinoma, AGS gastric carcinoma, HepG2 hepatocellular carcinoma, MCF-7 human breast adenocarcinoma was selected as the research cell line for the specific target of estrogen receptor (ER). Estrogen receptor, overexpressed in 70% breast cancer, has become the main therapeutic target in the treatment of ER-positive breast cancer cases. Nevertheless, structure and activity relationship of down-regulators of estrogen receptor has not been clarified and the clinical medicines for hormonal therapy are limited. It was hypothesized in this study that semi-synthesized DG01, DG03 and DG06 function as good ERDs based on the experimental screening data. The research was focused on the question whether DG series compounds could inhibit ER at protein and mRNA level and interfere with the ER-ERE binding activity. Moreover, signaling transduction involved in estrogen signaling was also studied to explain the comprehensive mechanism in depth. Further experiments made it clear that DG series compound enhance apoptotic effect by inhibition of ERα according to the comparison study of three breast cells.

Ⅱ. MATERIALS AND METHODS

2. 1. MATERIALS

2.1.1. Compounds

A series of steroid saponins (DG series) derived from diosgenin, bearing a unique α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranose moiety were synthesized by the research group of Prof. Mao-sheng Cheng, Shenyang Pharmaceutical Univ., Shenyang, China (Scheme 1-3.). The mechanism study was mainly conducted by using the most potential compound DG03, namely diosgenyl α-L-rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→4)]-α-L-arabinopyranoside.

2.1.2. Reagents and antibodies

RPMI1640 medium, Dulbecco's phosphate buffered saline (DPBS), dimethyl sulfoxide (DMSO), protease inhibitor cocktail, 4,6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and fulvestrant (ICI 182,780) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V-FITC and propidium iodide fluorescence microscopy kit were acquired from BD Biosciences (San Diego, CA, USA). Bovine serum albunin (BSA) was obtained from Pierce (Rockford, IL, USA). Epidermal growth factor was purchased from R&D Systems (Minneapolis, MN, USA). Primary and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Epitomics Inc. (Burlingame, CA, USA). The used primary antibodies are ERα, procaspase-3, procaspaes-8, PARP, Fas-L, Cyclin D1, c-Myc, p-Src, Src, Akt, p-Akt, mTOR, p-mTOR, ERK1/2, p-ERK1/2, p38, p-p38 and β-actin.

2.1.3. Cell culture

MCF-7 human breast adenocarcinoma, MDA-MB-231 human breast adenocarcinoma and MCF-10A human mammary epithelial cells are purchased from the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells and MDA-MB-231 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL). MCF-10A cells were cultured in DMEM supplemented with 5% horse serum, 1% penicillin and streptomycin, 10 μg/mL EGF and 20 ng/mL IGF. Cells were maintained in 5% CO_2 humidified atmosphere at 37℃.

2.2. METHODS

2.2.1 Cell cytotoxicity assay

Cell viability was assessed by MTT assay. MCF-10A, MDA-MB-231 and MCF-7 cells were seeded onto a 96-well plate at a density of 2×10^4 per well and incubated at 37℃ for 24 h. Cells were treated with various concentrations (0, 20, 40, 60 μM) of DG (00-12) series samples and incubated for an additional 24 h. 100 μL of MTT solution (0.5 mg/mL) was added into each well for 3 h and absorbance was measured at 540 nm using a EMax® microplate reader (Molecular Devices, CA, USA)

2.2.2. Observation of cell morphology and DAPI staining

MCF-10A, MCF-7 and MDA-MB-231 cells were grown in 24-well plates and incubated with DG03 under the condition of various concentrations and incubation times. After stained by 1 mM DAPI and washed with DPBS, cells were observed under CKX41 fluorescence microscope at the magnificence of 40 x 10.

2.2.3. DNA fragmentation assay

MCF-7 cells were seeded onto 6-well plates at a density of 4×10^5 per well and incubated at 37℃ for 24 h. Cells were treated with different concentrations (0, 10, 20, 30 μM) of DG03 and incubated for 24 h and 48 h respectively. After incubation, cells were harvested at centrifugation of 13,500 rpm for 5 min, washed with ice cold PBS buffer and re-centrifuged to collect cells. Total DNA was extracted by 200 μL Triton X-100 lysis buffer (50 mM Tris-Cl, pH 8.0, 200 mM EDTA, 5% Triton X-100), incubated on ice for 20 min. Lysates were

centrifuged at 14,000 rpm for 10 min, 4℃, and the supernatant was transferred to a new 1.5 mL tubes and extracted with 25:24:1 (v/v) phenol: chloroform: isopropyl alcohol (Sigma, USA) by gentle agitation for 5 min, RT. After centrifugation at 14,000 rpm for 10 min, 4℃, upper aqueous phase was transferred to a new 1.5 mL tube, adding 1/2 (v/v) of 10 M ammonium acetate and 2.5 times of total volume of ice cold absolute ethanol, and precipitated in -20℃ for overnight. DNA pellet was collected after centrifugation and re-suspended in 20 μL RNase solution (0.25 μg/mL), incubated at 37℃ for 30 min. DNA fragmentation assay was performed by electrophoresis with 2% agarose gel containing ethidium bromide (1 μg/mL) and the gel was photographed using Image Analysis software (UVP Inc, CA, USA).

2.2.4. Western blot analysis

MCF-7 cells (4×10^5 per well) were treated with concentrations of DG03 (0, 10, 20, 30 μM) for varied interval times (0, 6, 24, 48 h), lysed in Totex lysis buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM $MgCl_2$, 0.5mM EDTA) containing phosphatase and protease inhibitors and incubated on ice for 30 min with occasional mixing. Nuclear and cytosolic proteins were separated by using buffer C (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1.0 mM PMSF, 1.0 mM PI) and buffer A (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1mM PMSF, 1 mM PI). Measure protein concentration using Bradford reagent (Bio-Rad Laboratories Inc, CA, USA), ran up to 30 μg /lane on 8~12% polyacrylamide gel and electro-blotted to nitrocellulose membrane. After blocked with 5% skim milk for 1h at room temperature, membrane was incubated in various primary antibodies overnight followed by horseradish peroxidase-conjugated secondary antibodies. Membrane was developed using WEST-

ZOL® (plus) Western Blot Detection System (iNtRON Biotechnology Inc, Seoul, Korea) and target protein was visualized using LAS 1000 (Fuji film, Japan).

2.2.5. Semi-quantitative reverse transcriptase (RT)-PCR

MCF-7 cells (4×10^5 per well) were treated with concentrations of DG03 (0, 10, 20, 30 μM) for 24 h. Total RNA was extracted using easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology Inc, Korea) as suggested by manufacture. Nano-drop ND-1000 was used to quantify extracted RNA and cDNA was synthesized with reverse-transcription of 2 μg extracted total RNA employing RT Premix Kit (iNtRON Biotechnology Inc, Korea) according to manufacturer's instruction. The primers are: 5'-CGA CGC CAG GGT GGC AGA GAA AGA TT-3' (ERα forward), 5'-GGC CAA AGG TTG GCA GCT CTC ATG TC-3' (ERα reverse); 5'- TGC TGT TTC GAC GAC ACC GTT-3' (pS2 forward), 5'- AGG CAG ATC CCT GCA GAA GT-3'(pS2 reverse); 5'-TGA AGG TCG GTG TGA ACG GAT TTG GC-3' (GAPDH forward); 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3' (GAPDH reverse). The amplified cDNA was separated by 2% agarose gel containing ethidium bromide (1 μg/mL) and the gel was photographed using Image Analysis software (UVP Inc, CA, USA).

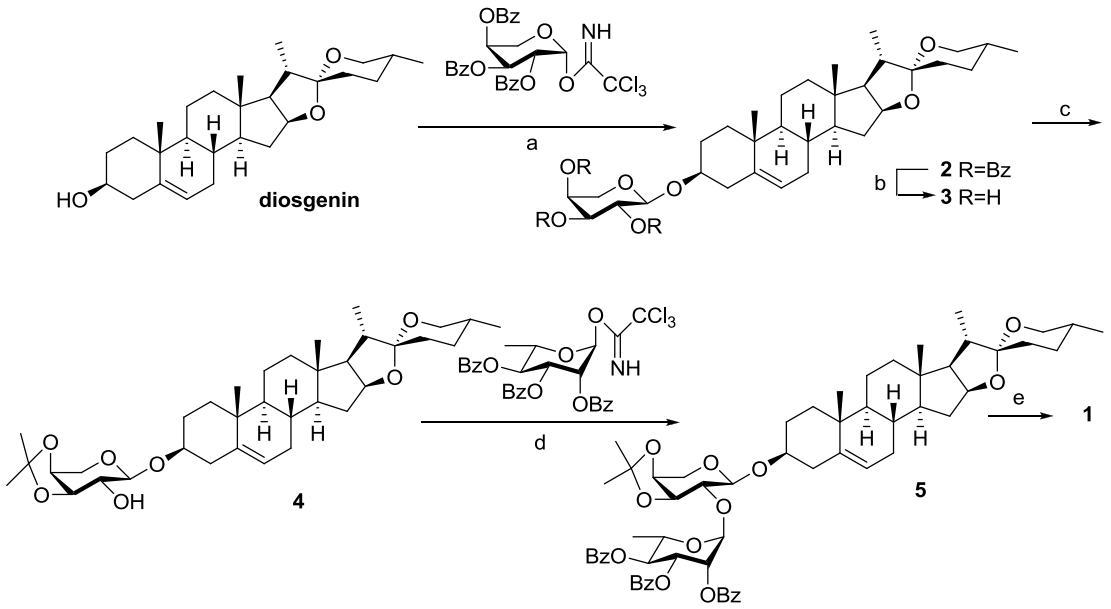
2.2.6. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts prepared from DG03 (0, 10, 20, 30 μM for 24 h) treated MCF-7 cells were incubated with ER consensus oligonucleotide (Santa Cruz, USA; sequence: 5'-GGA TCT AGG TCA CTG TGA CCC CGG ATC-3') and AP-1 consensus oligonucleotide (Promega, USA; sequence: 5'-ATT CGA TCG GGG CGG GGC GAG C-3') for 30min at 37℃, respectively. A 50-fold excess of unlabeled oligonucleotide was added to the reaction mixture as a competitor to verify the specificity for each binding site. DNA-protein complexes were

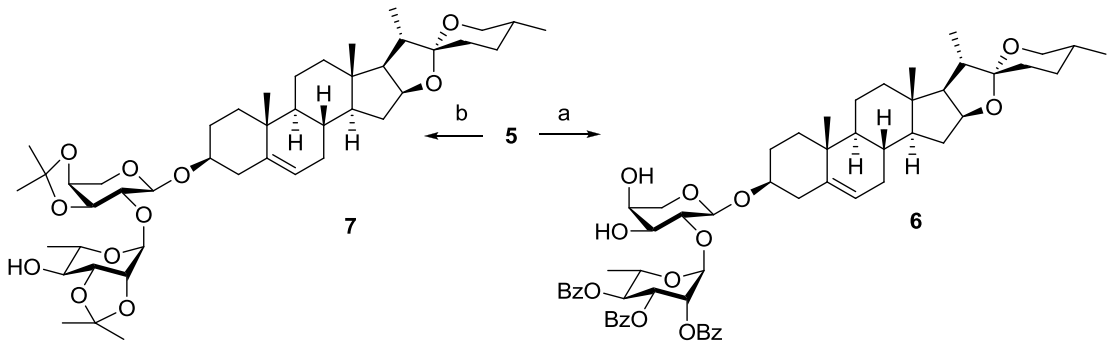
separated from the free oligonucleotides on 6% native polyacrylamide gels. The signals obtained from the dried gel were quantitated with an FLA-3000 apparatus (Fuji), using the BAS reader version 3.14 and Aida Version 3.22 software (Amersham Biosciences, USA). The binding conditions were optimized as reported earlier. [27]

2.2.7. Apoptosis assay: FACS and Flow cytometry

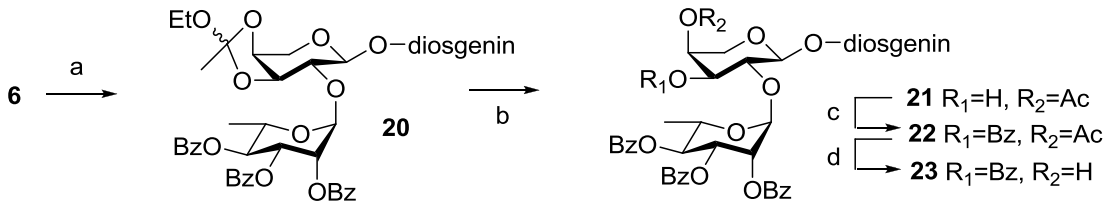
MCF-10A, MCF-7 and MDA-MB-231 cells were treated with 0, 10, 20, 30 μM of DG03 for 48 h. Cells were detached by using 1x trypsin EDTA (Biological Industries Ltd., Israel). After washing and collecting, cells were suspended in binding buffer and stained with FITC Annexin V and PI according to manufacturer's instruction of FITC Annexin V Apoptosis Detection Kit (BD Biosciences). Samples were analyzed on Flow cytometer (BD Biosciences) by using CellQuest Pro Software.



Scheme 1. Reagents and conditions of synthesis: (a) TMSOTf, CH_2Cl_2, 0 ℃, 1 hour, 90%; (b) NaOMe, CH_2Cl_2-MeOH, rt, overnight; (c) $Me_2C(OMe)_2$, TsOH, acetone, rt, 4h, 85% for two steps; (d) TMSOTf, CH_2Cl_2, rt, 15min, 80%; (e) TsOH, MeOH-CH_2Cl_2, 45℃; then NaOMe, rt, overnight, 65%(for two steps).



Scheme 2. Reagents and conditions of synthesis: (a) TsOH, MeOH-CH_2Cl_2, 45℃, 90%; (b) NaOMe, CH_2Cl_2-MeOH, rt, overnight; then $Me_2C(OMe)_2$, TsOH, acetone, rt, 2h, 75%(for over two steps).



Scheme 3. Reagents and conditions of synthesis: (a) $CH_3C(OEt)_3$, TsOH, CH_2Cl_2, rt; (b) 80% aq AcOH, rt, 82% (over two steps); (c) BzCl, pyridine, rt, 90%; (d) AcCl, CH_2Cl_2-MeOH, 0℃ to rt, <30%.

III. RESULTS

3.1. Cytotoxic effect of DG series compounds

Cell viability was inhibited by DG series compounds, among which DG01 and DG03 appeared to be the most toxic compounds bearing arabinose and xylose respectively, based on the IC_{50} values of each compound shown in four different cell lines, such as A549, HeLa, HepG2 and MCF-7 cells (Table 2). Cell viability in four cell lines didn't show much difference, but we chose to use breast cancer cell line MCF-7 because of its over-expression of ERα. The biological mechanism was studied by treatment of DG03 (Figure 7). Cell growth inhibition was tested by the treatment of DG03 in three different kinds of breast cell lines, such as MCF-10A, MCF-7 and MDA-MB-231, for 24 and 48 hours at different concentrations. The cell growth was more greatly inhibited in MCF-7 cells compared with other two breast cells (Figure 8). Effect of DG03 on MCF-7 cells in presence of ICI (780, 182) was tested to study the possible mechanism of DG03 (Figure 9)

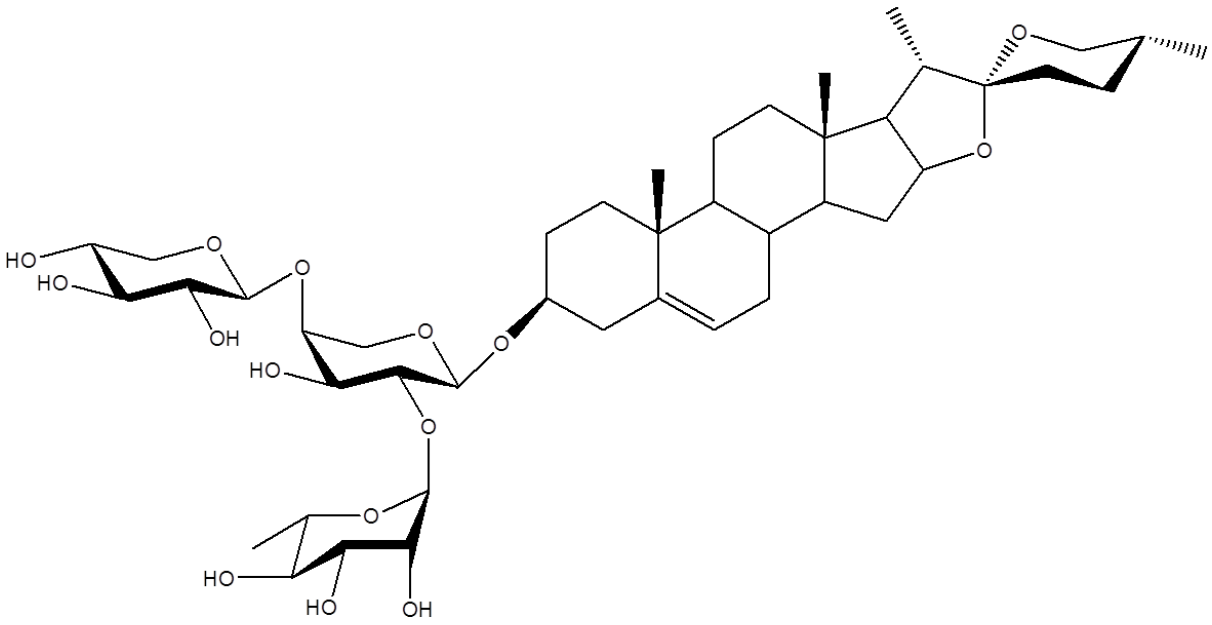


Figure 7. The structure of DG03

The mechanism study was mainly conducted by using the most potential compound DG03, namely diosgenyl α-L-rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→4)]-α-L-arabinopyranoside.

entry	compound	IC_{50} (μM)			
		A549	**HeLa**	**HepG2**	**MCF-7**
1	DG**00**	>60	>60	>60	>60
2	DG**01**	18.81	20.98	20.02	25.41
3	DG**02**	>60	>60	>60	>60
4	DG**03**	34.84	25.92	21.05	25.92
5	DG**04**	48.86	42.83	41.71	46.81
6	DG**05**	56.29	38.85	31.37	44.63
7	DG**06**	45.46	31.65	26.54	37.55
8	DG**07**	51.91	32.11	43.44	41.56
9	DG**08**	>60	>60	>60	>60
10	DG**09**	>60	>60	>60	>60
11	DG**10**	>60	>60	>60	>60
12	DG**11**	>60	>60	>60	>60
13	DG**12**	>60	>60	>60	>60

Table 2. IC_{50} values of DG series compounds of 4 human tumor cell lines in vitro

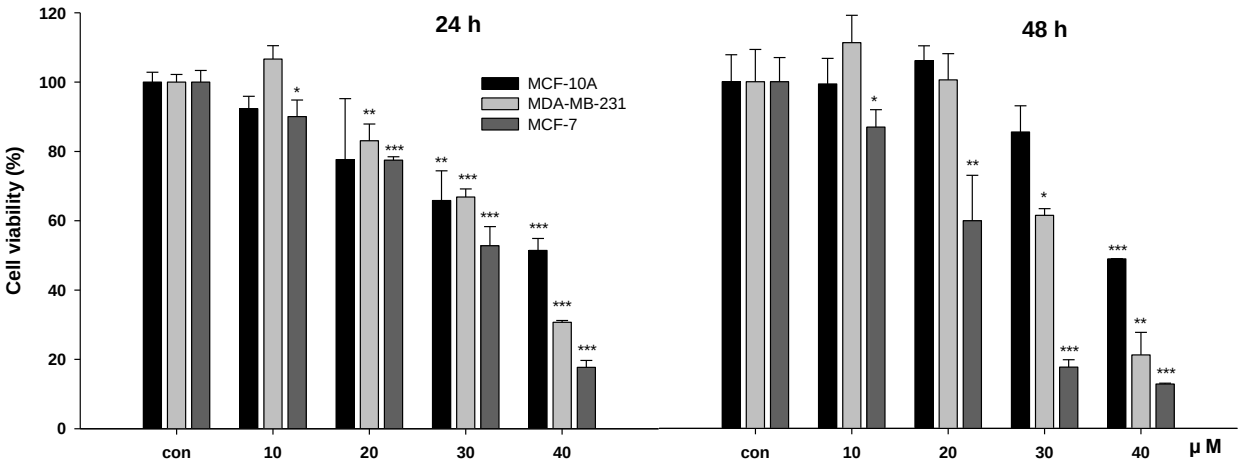


Figure 8. Cytotoxic effect of DG 03 on various breast cell lines

Cell viability was tested in three breast cell lines, such as MCF-10A, MCF-7 and MDA-MB-231. Cells were treated with different concentrations of DG 03 (10, 20, 30 and 40 μM) for 24 hours and 48 hours, respectively. Cell viability was determined by MTT assay.

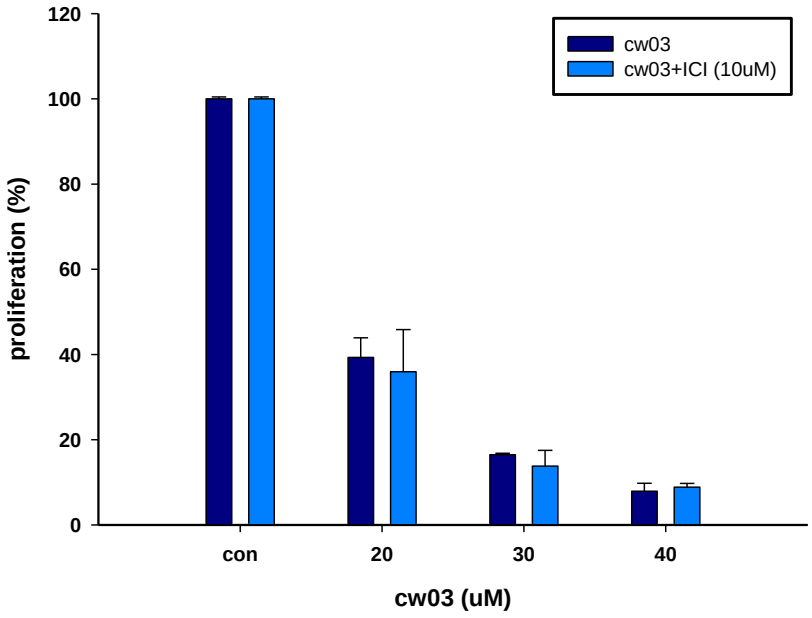


Figure 9. Effect of DG03 on cell proliferation in the presence of pure anti-estrogen ICI (182, 780)

Cells were also treated with DG03 with varied concentration from 20 to 40 μM in the presence of 10 μM ICI. Cells were treated for 24 hours and cell proliferation was determined by MTT assay.

3.2. Effects of DG03 on cell morphology and nuclear fragmentation

To examine the differences of apoptotic effect of DG03 on MCF-10A, MCF-7 and MDA-MB-231 cells, cell morphology observation, DAPI staining assay and DNA fragmentation were performed. Cell rounding and shrinkage were observed after the sample treatment of 24 hours (Figure 10). According to DAPI staining, cells produced apoptotic bodies and most of cells were detached because of cytotoxic and apoptotic effects (Figure 11). DNA fragmentation occurred dose dependently after 48 hours of sample treatment, however, it did not occur after 24 hours of treatment, indicating that apoptotic effect of DG03 began later than 24 hours (Figure 12). The experimental results showed that DG03 may induce apoptosis in MCF-7 breast cancer cells.

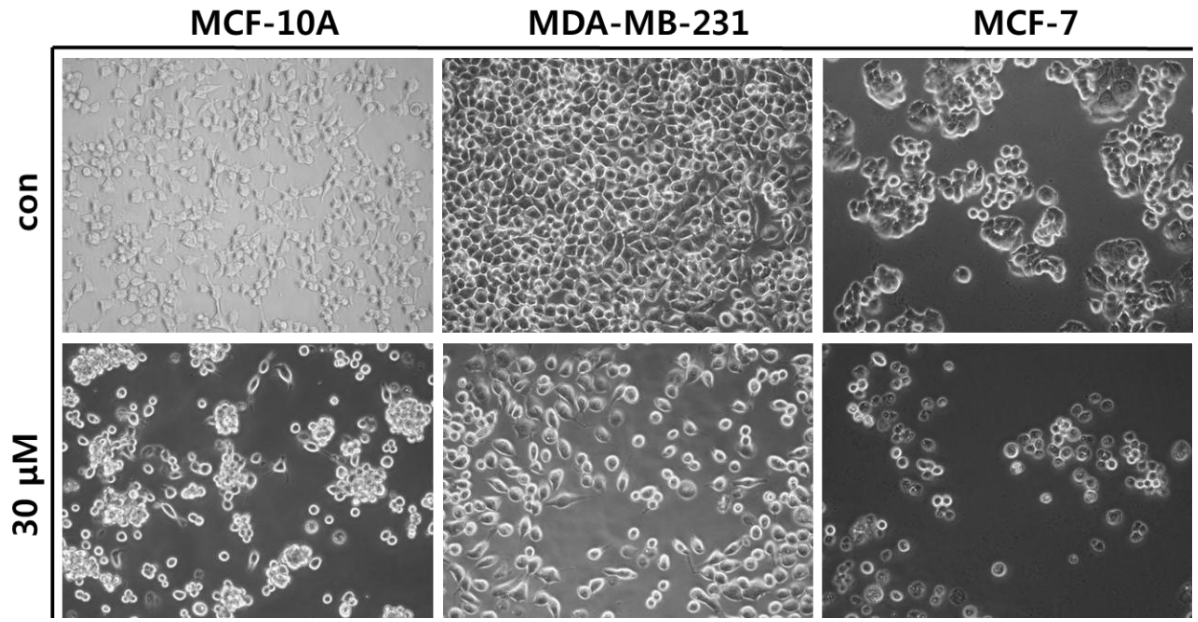


Figure 10. Observation of cell morphology in three breast cell lines

MCF-10A, MCF-7 and MDA-MB-231 cells were treated with or without DG03 (30 μM) and incubated for 24 hours to observe morphological change. Cell rounding could be observed in all three cell lines, while it was most significantly occurred in MCF-7 cell line.

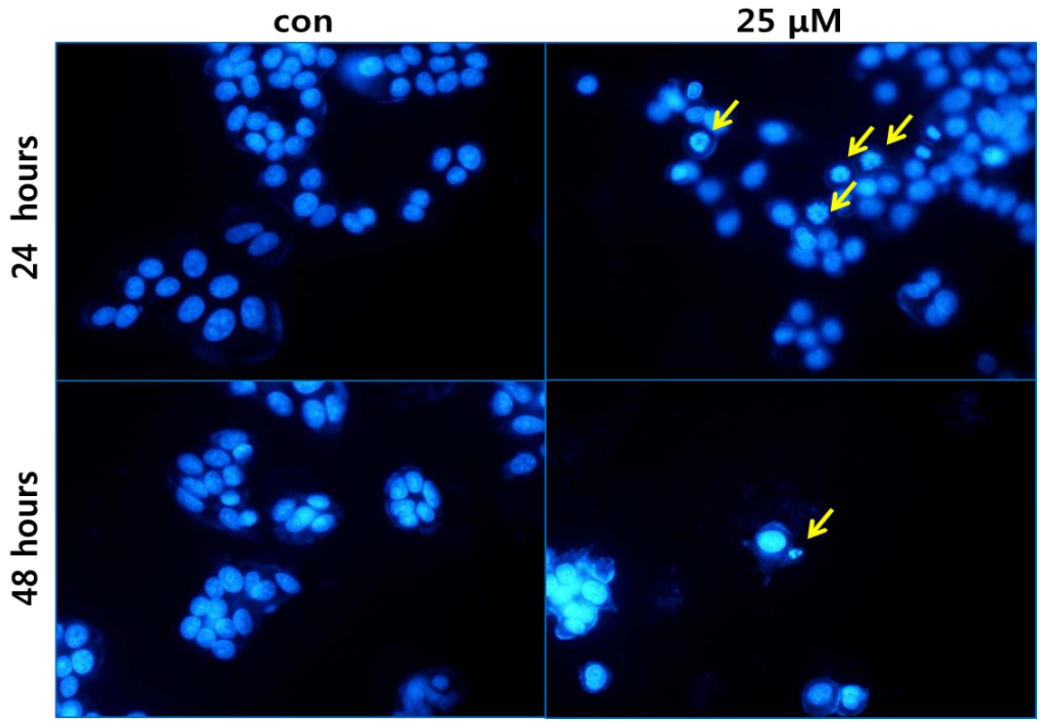


Figure 11. Observation of nuclear fragmentation by treatment of DG**03 in MCF-7 cells**

More apoptotic bodies were observed in 48 hours group than 24 hours group after the treatment of DG03 in MCF-7 cells.

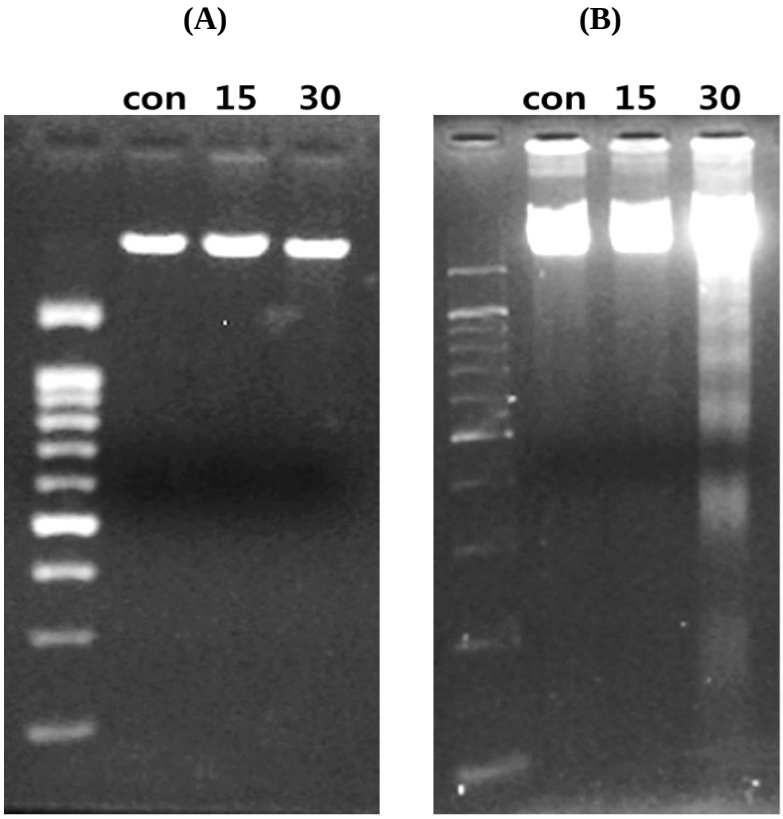


Figure 12. DNA fragmentation by treatment of DG03 in MCF-7 cells

MCF-7 cells were treated with 15 μM and 30 μM of DG03 for 24 hours (A) and 48 hours (B). DNA fragmentation occurred dose dependently after 48 hours of the treatment of DG03.

3.3. Apoptosis induced by treatment of DG03 in three breast cell lines

Flow cytometry analysis of FITC Annexin-V/PI double staining was conducted to verify the apoptotic effect of DG03 on three breast cell lines, such as MCF-10A, MCF-7 and MDA-MB-231 cells (Figure 13). MCF-7 cells showed most significant apoptotic effect compared with other two breast cell lines. In MCF-7, both of early and late apoptotic groups were greatly increased by treatment of DG 03 dose dependently, indicating that DG03 induces apoptosis selectively in MCF-7 human breast adenocarcinoma. In order to study the effect on other cancer cell lines, apoptotic effect was tested in A549 lung cancer cell line, but apoptotic effect was much lower than that in MCF-7 cell line (Figure 14)

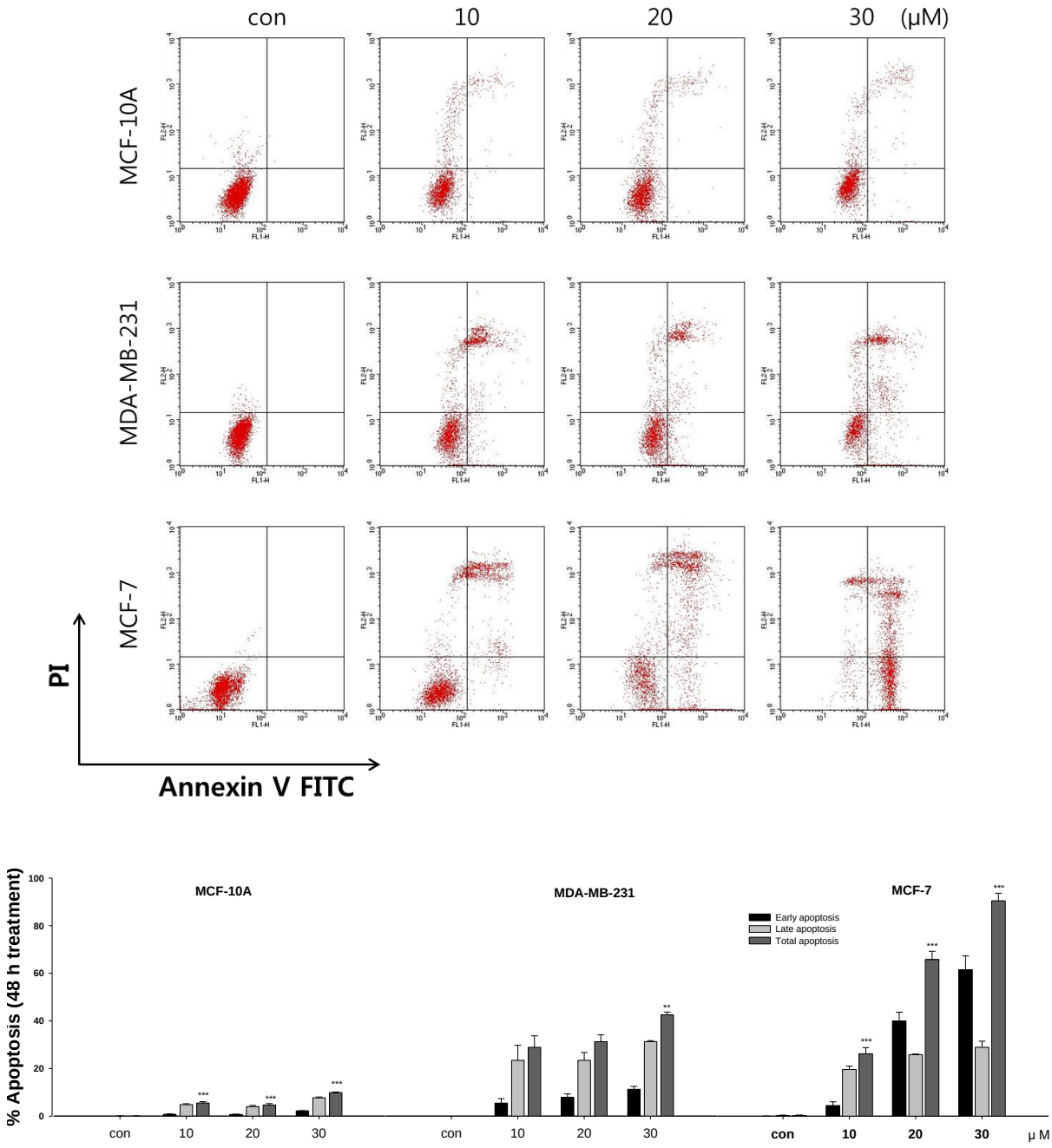


Figure 13. Flow cytometric analysis of Annexin-V/ PI double staining in breast cell lines

Flow cytometric analysis of Annexin-V/ PI double staining was performed in MCF-10A, MCF-7 and MDA-MB-231 cells after 48 hours incubation with various concentrations of DG03 (0, 10, 20, 30 μM).

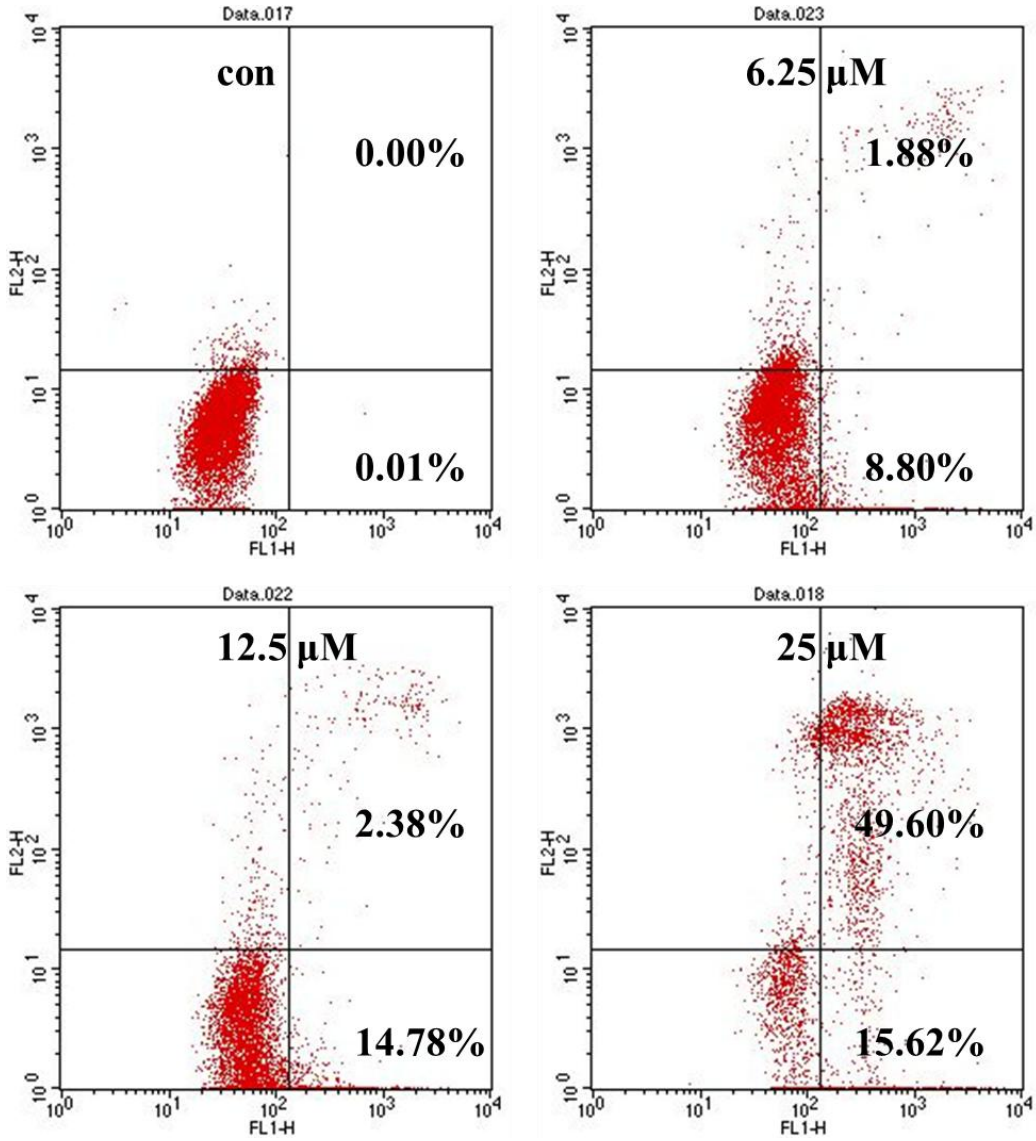


Figure 14. Flow cytometric analysis of Annexin-V/ PI double staining in A549

Flow cytometric analysis of Annexin-V/ PI double staining was performed in A549 cells after 48 hours incubation with various concentrations of DG03 (0, 6.25, 12.5, 25 μM).

3.4. DG series decreases protein and mRNA level of ERα in MCF-7 cells

Diosgenin, the backbone structure of DG series compounds, is a well-known compound used for the synthesis of steroid products, such as progesterone. Nevertheless, the pro-estrogenic activity of diosgenin converted to anti-estrogenic activity by introducing sugar groups at C-3 [28]. Estrogen receptor positive cells, MCF-7 were treated with 25 μM of each compound and the protein level of ERα was detected by Western blotting (Figure 15). Among six cytotoxic DG series compounds, DG03 showed most potent effect on reducing ERα compared to positive control (ICI 182,780). On the other hand, non-glycosylated compound DG00 didn't show any inhibition effect on ERα. ERα expression in MCF-7 cells reduced in dose- and time- dependent manner when treated with DG03 (Figure 16A). As a member of the nuclear receptor family, ERα functions as a transcriptional factor when activated by estrogen in nucleus. DG03 decreased ERα protein expression in both of nuclear and cytosolic fraction of extracted protein (Figure 16B). In order to study whether the compound inhibits ERα expression at transcriptional level, semi-quantitative reverse transcriptase (RT)-PCR was conducted and mRNA of ERα was found out to be inhibited significantly at 30 μM (Figure 16C).

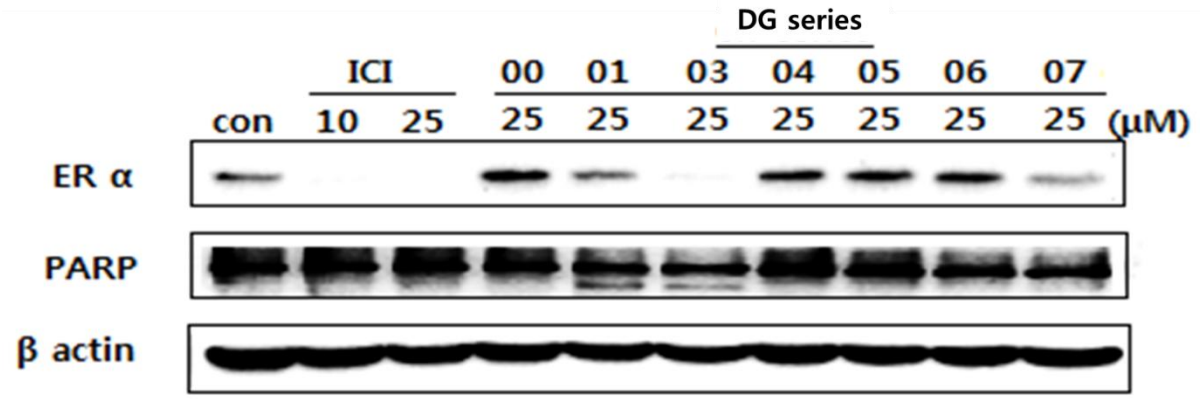
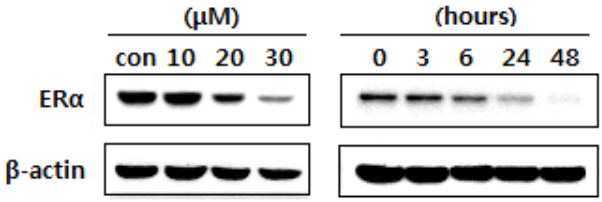


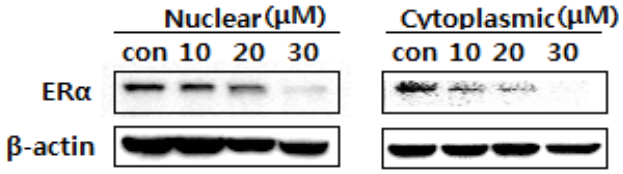
Figure 15. Inhibition effect of DG series compounds on ERα and PARP

Six of twelve DG series compound were tested for ERα inhibition and PARP cleavage by Western Blot. Cells were treated with 25 μM of each compound for 24 hours and ICI 182,780 (10 and 25 μM) was used as a positive control.

(A)



(B)



(C)

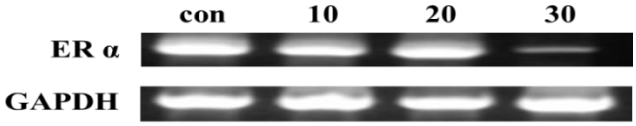


Figure 16. DG series compounds inhibit protein and mRNA expression of ERα in MCF-7 cells

(A) DG03 decreases ERα protein expression in dose and time dependent manner. Cells were treated with various concentrations of DG03 (0, 10, 20, 30 μM) for 24 hours and 30 μM of DG03 for the indicated time points. **(B)** DG03 decreased ERα protein expression in nuclear and cytoplasmic extract. Cells were treated with various concentrations of DG03 (0, 10, 20, 30 μM) for 24 hours and ERα expression was detected in both nuclear and cytoplasmic portion separately. (A-B) were tested by Western blot. **(C)** DG03 decreased mRNA of ERα in MCF-7. Cells were treated with various concentrations of DG03 (0, 10, 20, 30 μM) for 24 hours. Total RNA was extracted and mRNA of ERα was detected by RT-PCR.

3.5. DG03 inhibited ER-ERE binding activity through ER-dependent classical and non-classical pathway

Transcriptional activity of ERα can be initiated by direct binding of ERα to estrogen responsive element (ERE) or indirect binding of ERα to promoter regions of some genes with other transcription factors, such as AP-1 [6]. ERα mediated activation of genes, including pS2, cyclin D1, c-Myc etc, is regulated by recruitment of ERα to their promoter regions [11]. To investigate the DNA binding activity of ERα and AP-1 in DG03 treated MCF-7 cells, Electrophoretic mobility shift assay (EMSA) was performed (Figure 17). Competition assay was performed to measure the specificity of binding. ER-ERE binding activity was inhibited dramatically according to the results, indicating that the repression of ERα expression leads to decreasing of ER-ERE interaction. On the other hand, ERα mediated AP-1 binding affinity was also decreased dose-dependently, which elucidates that DG03 induced apoptosis confronted with ER classical and non-classical signaling pathways. Based on further investigation, expression level of pS2, cyclin D1 and c-myc were decreased dose dependently as supposed (Figure 18). Phosphorylation of tyrosine kinase Src was inhibited, indicating that signaling transduction of ERα was inhibited by the treatment of DG03 (Figure 18B).

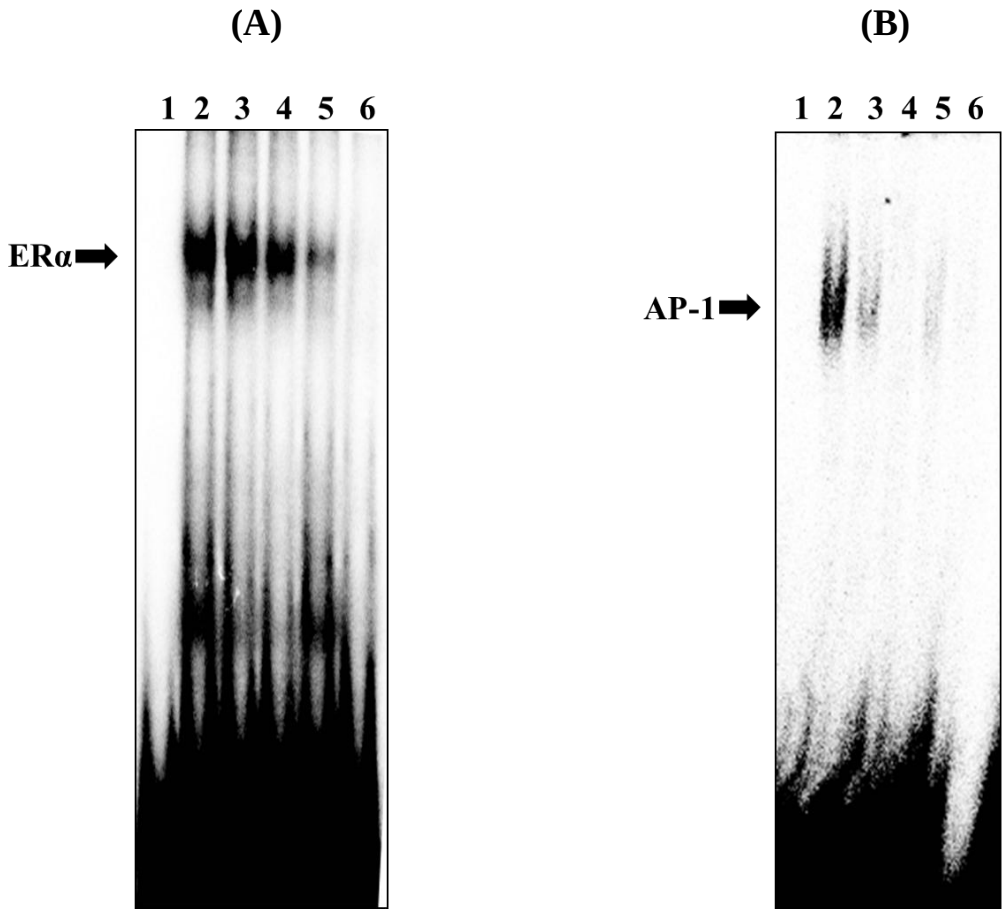
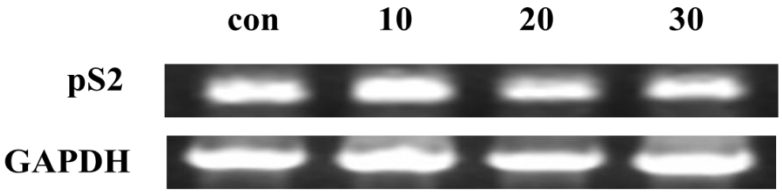


Figure 17. Inhibition of ER-ERE binding activity

DNA binding activity of ERα and AP-1 at 24 hours' time point was investigated by using EMSA. **(A)** ERE consensus oligonucleotide. **(B)** AP-1 consensus oligonucleotide. Each lane stands for 1. probe, 2. control, 3. DG03 10 μM, 4. DG03 20 μM, 5. DG03 30 μM, 6. Competition assay.

(A)



(B)

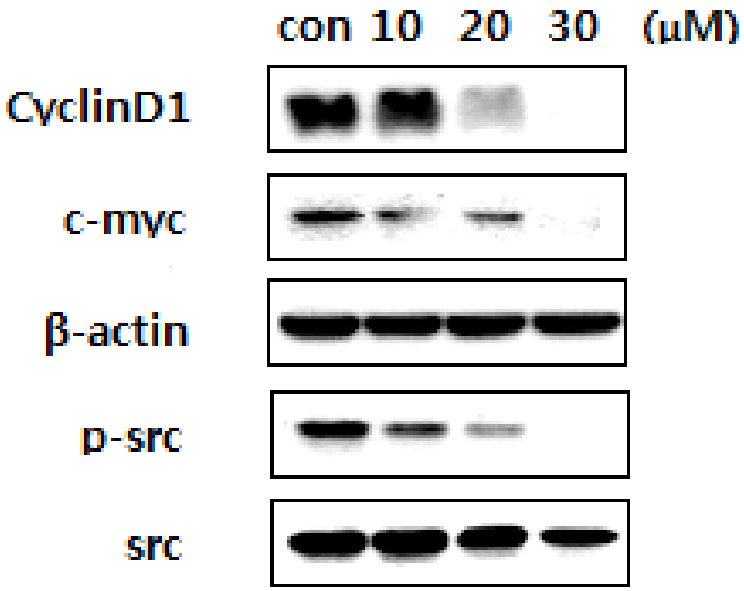


Figure 18. Suppression of ER induced transcriptional gene expression

(A) ER induced mRNA expression of pS2 was measured by RT-PCR and GAPDH was used as loading control. **(B)** Cells were treated with various concentrations of DG03 (0, 10, 20, 30 μM) and incubated for 24 hours. Expression of cyclin D1 and c-Myc were detected by Western Blotting. Phosphorylation of Src was inhibited dose dependently.

3.6. DG 03 induced apoptosis through extrinsic death receptor pathway

According to Western blotting analysis, DG03 inhibited apoptotic proteins of procaspas-3, procaspase-8 and PARP (Figure 19). Survivin, a member of the inhibitor of apoptosis (IAP) family, was inhibited. However, pro-caspasase-9 and Bcl-2 remained at the same level even at the high concentration, and Fas ligand was up-regulated dose dependently. Hence, it can be demonstrated that DG03 induced apoptosis through extrinsic death receptor signaling pathway but not intrinsic signaling pathway. Comparison study was also conducted by treatment of DG01, DG03, DG04 and DG05 (Figure 20). As a result, apoptotic proteins were inhibited significantly in DG01 and DG03 in accordance with cell viability result.

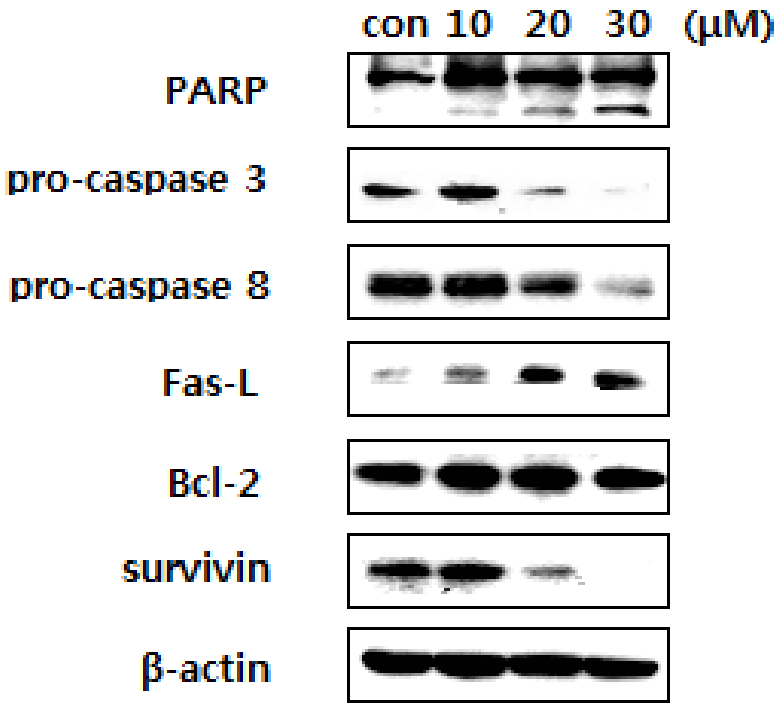


Figure 19. Effect of DG03 on apoptotic protein expression

Cells were treated with various concentrations of DG03 (0, 10, 20, 30 μM) for 48 hours and Western Blotting was performed with extracted total protein. Indicated antibodies, such as procaspase-3, procaspase-8, Fas-L, Bcl-2, survivin and PARP, were used.

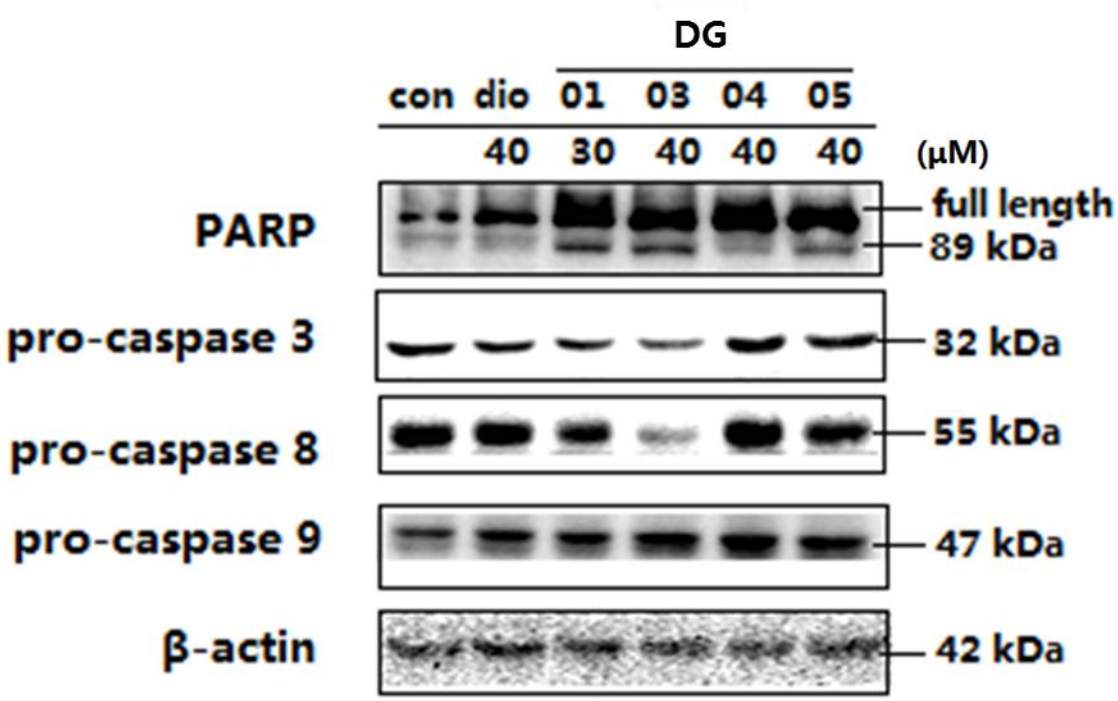


Figure 20. Comparison study of apoptosis of DG01, DG03, DG04 and DG05

Comparison study was conducted by treatment of diosgenin, DG01, DG03, DG04 and DG05. Indicated antibodies, such as procaspase-3, procaspase-8, procaspase-9 and PARP, were used.

3.7. DG03 inhibited AKT and MAPK signaling pathways

It has been noticed that apoptosis could be prevented through estrogen receptor signaling pathways, in which phosphoinositide 3-kinase (PI3-kinasae) pathways are involved [29]. Phosphorylation of p-mTOR and p-AKT was inhibited dose dependently according to our data (Figure 21). Another ERα non-genomic signaling MAPK pathway was inhibited by DG03 according to Western blotting data of p-p38/p-ERK1/2 (Figure 22).

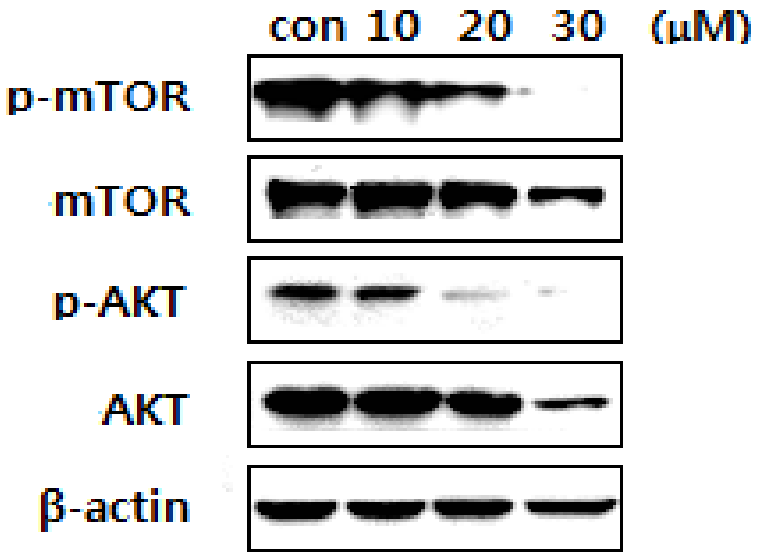


Figure 21. Effect of DG03 on AKT signaling pathway

Cells were treated with various concentrations of DG03 (0, 10, 20, 30 μM) for 48 hours and Western Blotting was performed with extracted total protein. P-mTOR and p-Akt were detected.

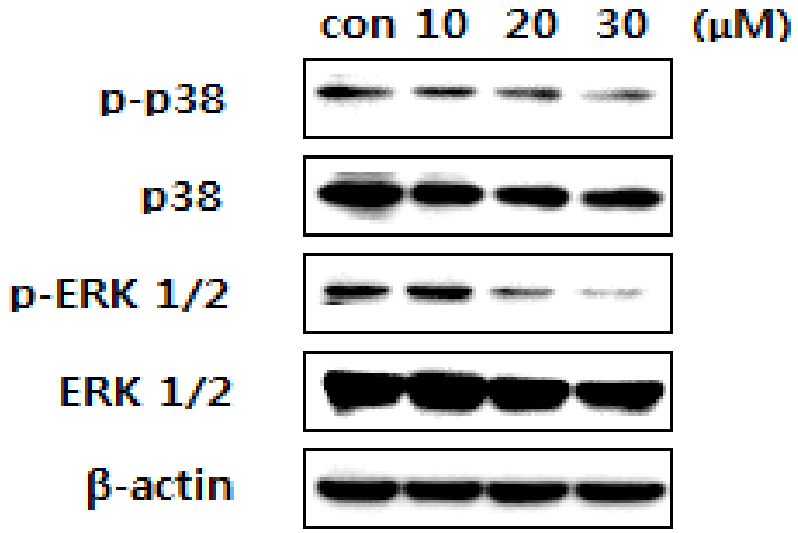


Figure 22. Effect of DG03 on MAPK signaling pathway

Cells were treated with various concentrations of DG03 (0, 10, 20, 30 μM) for 48 hours and Western Blotting was performed with extracted total protein. P-p38 and p-ERK 1/2 were detected.

Ⅳ. DISCUSSION

Worldwide, breast cancer is the most common cancer diagnosed in women and survival rates are still much poorer in developing countries. Hormonal therapy, also known as anti-estrogen therapy, is the main choice of treatment against estrogen receptor positive breast cancers [30]. Estrogen receptor alpha (ERα) plays a vital role in breast cancer development and progression due to its transcriptional activity in gene expression of cell cycle regulators and growth factors [13, 31]. Knockdown of ERα in ER-positive breast cancer can inhibit cell proliferation and result in apoptosis by deletion of up-regulation effect of anti-apoptotic protein Bcl-2 [32, 33]. Moreover, protein-protein interactions with cell cycle regulator proteins, such as p27 cdk, arrest cells in S phase to induce apoptosis [13]. Hence, it was demonstrated in our study that a potential ERα down-regulator DG03, which showed best ERα inhibitory effect among thirteen semi-synthesized DG series compounds, induces apoptosis in ER-positive MCF-7 breast cancer cells by estrogen receptor signaling pathway.

In this study, anti-tumor activity of semi-synthesized DG03 was tested in three breast cell lines. It was found that DG03 showed more significant anti-proliferative effect on ERα-positive MCF-7 cancer cells compared with non-tumorigenic epithelial MCF-10A and ERα-negative MDA-MB-231 cells. According to the flow cytometric analysis of apoptosis in three breast cell lines, DG03 showed apoptotic activity in all three cell lines but most prominent activity in MCF-7 cells. It can be indicated that DG03 has ERα-targeted anti-tumor activity in breast cancer. Six of thirteen DG series compounds showed cytotoxic activity while only three of them functioning as potential down-regulators of ERα when pure anti-estrogen fulvestrant (ICI 182,780) was used as a positive control. In MCF-7 human breast cancer cells,

each of nuclear and cytosolic protein expression of ERα was suppressed dose and time dependently after DG03 was treated. The effects of DG03 on MCF-7 cells are probably due to down-regulation of ERα transcriptional activity through classical and non-classical ER-ERE binding, which resulted in reduction of ERα responsive gene expressions, such as cyclin D1, c-Myc and pS2. Cyclin D1 plays a key role in G1-S phase entry and proliferation through its interaction with ERα in breast cancer development, indicating that suppression of cyclin D1 led to the inhibition of cell proliferation and cell cycle arrest [13]. In addition, the ERα-mediated protein reduction of c-Myc contributed to cellular apoptosis and cell cycle arrest and the inhibition of pS2 demonstrated the inactivation of ER signaling [34, 35]. Moreover, decreased binding activity of ERα and AP-1 on their DNA binding sites supplemented additional data that DG03 inhibited ERα protein and mRNA expression and suppressed estrogen receptor signaling through classical and non-classical pathway in ER-positive MCF-7 cells.

Apart from ligand-dependent transcriptional action of ERα, it has been reported that a group of membrane-bound ERα is non-genomic and activates the Src/ERK pathway that triggers cell proliferation and differentiation [36, 37]. Non-receptor tyrosine kinase Src, observed in up to 40% of ER-positive cancers, link a variety of extracellular signals to critical intracellular signaling pathway [38, 39]. Plasma membrane bound ERα initiate Src/ERK phosphorylation cascade by association with Src for the incapability of kinase activity of ERα [36]. It has been reported that inhibition of ERK1/2 enhanced tamoxifen-mediated growth inhibition in tamoxifen-resistant MCF-7 breast cancer cells [40]. Analysis of our data indicates that DG03 suppresses ERα-mediated non-genomic signaling through inhibition of phosphorylation of Src and p38/ERK of MAPK pathway, resulting in cell signaling growth

inhibition and induction of apoptosis. On the other hand, Src also play a central role in activation of phosphatidylinositol-3-kinase (PI3K)/AKT pathway [41]. Previous study has reported the high expression of p-Akt in breast cancer and activation of PI3K/AKT pathway contributes to breast cancer tumorigenisis [42, 43]. Additionally, combination of LY294002, PI3K inhibitor, with tamoxifen in ER-positive cells greatly potentiated apoptosis [43]. Here, inactivated PI3K/AKT signaling accords with reduced phosphorylation of Src, potentiates the disruption of ERα-mediated non-genomic signaling and further signaling transduction of apoptosis.

Two major pathways that lead to apoptosis have been identified in this study. Apoptosis related protein level, such as caspases-3 and caspase-8, were reduced without suppressing caspase-9, which is initiated during intrinsic pathway. Supportively, no inhibition effect was achieved when Bcl-2 was detected and Fas Ligand, a trans-membrane protein part of TNF family, was activated by treatment of DG03. Active caspase-3 was not detected for the possible reason that it was reported to be not expressed in MCF-7 cell lines [44]. Inhibition results of caspase-3 and caspase-8 were correlated well with the cleavage of PARP, indicating that DG03 induced apoptosis in MCF-7 cells [45]. Further study of survivin, a member of inhibitor of apoptosis (IAP) family, demonstrated that DG03 induces apoptosis by removing inhibition effect of survivin on cysteine-aspartic proteases [46].

In conclusion, DG series compounds, DG03 especially may interfere with ERα-mediated classical and non-classical transcriptional signaling by inhibition of protein and mRNA of ERα, as well as ER-ERE binding activity. Moreover, the extracellular reduction of non-genomic ERα signaling involves inhibition of AKT/MAPK pathway and induces apoptosis through extrinsic pathway in ER-positive MCF-7 breast cancer cells.

ABSTRACT IN KOREAN

디오스게닌 유도체에 의한 MCF-7 유방암 세포의 항암활성연구

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본 연구에서는 합성화합물인 디오스게닌 (diosgenin) 의 배당체 (DG 시리즈)가 유방암 세포인 MCF-7 세포에서 과발현되는 에스트로젠 수용체를 특징적으로 억제시킴으로써 세포사멸을 일으킨다는 가설을 두고 실험을 계획하였다. 에스트로젠 수용체는 70 퍼센트 이상의 유방암에서 과발현되며 유방암, 대사질환, 골다공증 등과 같은 질병의 진단에 쓰인다. 임상에서 에스트로젠 수용체는 유방암 치료에서 효과적인 치료 표적이 될 수 있다. 디오스게닌 배당체들은 디오스게닌과 베타헤데린 (β-hederin) 에서 유래된 이당 구조 (α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranose)를 결합시키고 각각의 특징적인 단당을 결합시켜 얻은 화합물들이다. DG 시리즈 화합물들은 총 13 가지 (DG00-12)를 합성 하였으며 그 중 DG01, DG03 과 DG06 등 화합물이 MCF-7 유방암세포에서 세포독성과 에스트로젠 수용체의 억제효과를 나타내었다. 실험을 통해 DG03 의 에스트로젠

수용체 억제 효과가 가장 좋으며 이는 단백질과 mRNA 의 수준에서 에스트로젠 수용체의 발현을 억제한다는 결과를 얻었다. 에스트로젠 수용체의 전사인자로써의 활성을 측정하기 위하여 Electrophoretic mobility shift assays (EMSA) 를 통하여 에스트로젠 수용체의 DNA 결합능력을 측정하였다. 그 결과 DG03 은 에스트로젠 수용체의 DNA 결합능력을 억제 할 뿐만 아니라 에스트로젠 수용체의 전사산물인 c-Myc, cyclin D1, pS2 등 인자들의 발현을 억제하는 것으로 나타났다. 또한 에스트로젠 수용체에 의한 세포신호전달 과정은 AKT 와 MAPK 등 과정을 거쳐서 신호전달이 이루어 지며 이는 최종적으로 세포사멸을 일으킨다. Fluorescence-activated cell sorting (FACS)를 이용하여 MCF-7, MCF-10A, MDA-MB-231 등 세가지 유방세포에서 세포사멸효과를 측정한 결과 에스트로젠 수용체를 과발현하는 유방암 세포인 MCF-7 세포에서 가장 좋은 세포사멸효과를 얻을 수 있었다. 위 실험결과에 근거하여 DG03 은 유방암 세포에서 세포사멸을 일으키며 이는 특징적으로 에스트로겐 수용체를 표적으로 한다는 결론을 내릴 수 있다. 즉 디오스게닌의 유도체인 DG03 은 선택적 에스트로젠 수용체 하향조절제 (SERDs)의 역할을 하고 세포사멸을 일으키므로 에스트로젠 수용체를 과발현하는 유방암 치료의 후보시료로 사용할 수 있다.

주요어: 에스트로겐 수용체, diosgenin 배당체, 세포사멸, MCF-7, ER signaling, AKT/MAPK pathway
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Omer, I certainly believe that you are a good scientist and I wish you will be a good supervisor and realize your dream in the near future. Thank you very much.

재무 오빠, 처음 실험실에 들어 왔을 때 오빠 옆자리에 앉아 오빠한테 이것 저것 질문 했을 때가 그립습니다. 실험실 생활에 적응 못하고 방황 할 때에도 많은 조언을 주었고 아무 것도 모르는 저에게 하나부터 열까지 친절하게 설명 해주던 재무 오빠에게 항상 감사하다는 말씀을 전하고 싶었습니다. 감사합니다.

서미영, 대학교와 대학원을 모두 같은 학교에서 공부하게 된 미영이랑 나는 인연이 깊은 것 같아. 더 많은 시간을 함께 할 수 없게 된 것 같아 아쉬울 뿐이야. 앞으로 훌륭한 연구자가 되어 너의 역량을 충분히 발휘 할 모습을 기대 할께. 고맙고 보고 싶을 거야.

박샛별, 언니의 옆자리에 앉아 4차원식의 개그를 주고 받을 때가 즐거웠습니다. 연구를 즐기는 모습이 보기 좋았고 앞으로 좋은 성과가 있길 바랍니다. 감사합니다.

이경진, 경진이랑은 가장 많은 시간을 보낸 것 같아. 너랑 웃고 장난치던 나날이 앞으로 더욱 그리워 질 거야. 하나 밖에 없는 후배인데 더 잘해주지 못하고 항상 도움만 받아서 고맙고 미안한 마음이 들어. 앞으로 멋진 연구자가 되길 진심으로 바랄께.

김보은, 밝고 씩씩한 언니의 모습이 보기 좋았습니다. 남은 대학원 생활을 잘 마무리 하기를 바랍니다. 감사합니다.

송다영, 졸업 할 시기에 만나게 되어 아쉽지만 엉뚱한 다영이 때문에 즐거웠던 것 같아. 앞으로 실험실 생활을 잘 하길 바랄께.

정호 오빠와 윤영이, 두 분은 언제나 저의 부족한 부분을 채워줬던 것 같습니다. 상냥하고 친절하신 정호 오빠에게 행운이 찾아 오기를 바라겠습니다. 윤영이도 부디 하고 싶은 일이 잘 되고 항상 밝은 모습을 보여 줬으면 좋겠습니다. 두 분께 진심으로 감사 드립니다.

감사의 글을 마무리 하면서 마지막으로 먼 중국에 계신 저의 사랑하는 부모님께 감사의 말씀 드리고 싶습니다. 앞으로 더욱 멋진 딸이 되겠습니다. 감사합니다.

2012년 07월 04일