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Proteasome-independent down-regulation of estrogen receptor-α (ERα) in breast cancer cells treated with 4,4′-dihydroxy-*trans*-stilbene

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ABSTRACT

Treatment of cells with estrogens and several pure ERα antagonists rapidly induces down-regulation of the α-type estrogen receptor (ERα) in the nucleus by mechanisms that are sensitive to the proteasome inhibitors, MG132 and *clasto*-lactacystin-β-lactone. Hence, it is believed that these ER ligands induce down-regulation of ERα by proteasome-dependent mechanisms, which serve to control both the amount of transcriptional activity and the level of ligand-bound ERα in cells. In this study, we observed that treatment of cultured MCF-7 and T47D human breast cancer cells with the low affinity ER ligand, 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), inhibited the transcriptional activity of ERα and induced slow and gradual decrease in the amount of ERα protein (henceforth referred to as down-regulation of ERα). The 4,4′-DHS-induced down-regulation of ERα in MCF-7 cells involved a mechanism that was insensitive to the two most specific proteasome inhibitors, *clasto*-lactacystin-β-lactone and epoxomycin, but sensitive to MG132 at concentrations exceeding that required for maximal inhibition of the proteasome in MCF-7 cells. Therefore, 4,4′-DHS appears to induce down-regulation of ERα by a proteasome-independent mechanism. Here, we present data to show that both 4-OH and 4′-OH are critical for the ability of 4,4′-DHS to induce down-regulation of ERα and suggest that 4,4′-DHS provides a useful scaffold for development of novel ERα antagonists.

1. Introduction

Estrogens are essential for the development and function of numerous tissues, most notably the brain, bone, the cardiovascular system and female reproductive tissues such as the breast and uterus [1]. At the molecular level, the effect of estrogens on cells is primarily mediated by ERα and the β-type estrogen receptor (ERβ) in the nucleus. ERα and ERβ share

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six homologous regions and function as estrogen-dependent transcription factors [2,3]. Binding of estrogen to the ligand-binding domain (LBD) of the ERs causes conformational changes in and activation of the receptors, and formation of receptor homo-dimers, which bind to the *cis* estrogen-responsive elements in the promoters of estrogen-responsive genes [2,3], and stimulate the expression of these genes in the presence of co-activators [4,5]. The estrogen-responsive genes encode the proteins that regulate various estrogen-dependent cellular activities, and hence, regulation of the transcriptional activity and the amount of the ERs is fundamental to the control of the estrogen-dependent activities in cells.

The eukaryotic proteasome is a giant 26S ATP-dependent proteolytic complex, which possesses chymotrypsin-like, trypsin-like and peptidyl glutamyl peptide hydrolase (PGPH) activities that play a major role in the degradation of all short-lived and many long-lived nuclear and cytosolic proteins [6,7]. Therefore, the proteasome is directly or indirectly involved in the regulatory processes of a myriad of cellular activities, including signal-transduction, cell cycle progression, cell proliferation, differentiation and apoptosis [6,7]. Prior to being degraded by the proteasome, a target protein of the proteasome is ubiquitinated, that is, it is covalently modified at a specific lysine residue by a polymer chain of ubiquitin. Ubiquitination of the protein essentially marks the protein for the proteasome to recognize and degrade it [6,7].

Therefore, accumulation of ubiquitinated proteins and up-regulation of various target proteins of the proteasome in cells are indications of reduced activity of the proteasome in the cells.

It has been demonstrated that ERα can be ubiquitinated and degraded by the proteasome in vitro [8], and treatment of various types of cells with the natural estrogen, 17-β-estradiol (E_2), rapidly induces down-regulation of ERα by mechanisms that are sensitive to the proteasome inhibitors, MG132 and *clasto*-lactacystin-β-lactone [8–15]. Furthermore, E_2 induces down-regulation of the wild-type ERα, but not a mutant ERα that lacks ligand-binding activity [11]. Therefore, degradation of the E_2-occupied ERα by the proteasome is responsible for down-regulation of ERα in cells treated with E_2. Also, it has been demonstrated that treatment of cells with various pure ERα antagonists, including ICI-182-780 and GW5638, rapidly induces down-regulation of ERα by mechanisms that are sensitive to MG132 and *clasto*-lactacystin-β-lactone [11–13,16], and that binding of the antagonists to ERα is a prerequisite for these antagonists to induce down-regulation of ERα [11]. Therefore, binding of either an agonist or a pure ERα antagonist to ERα marks it for ubiquitination and subsequent degradation by the proteasome. Currently, most aspects of the proteasome-dependent mechanisms involved in down-regulation of ERα occupied by ligands remain virtually uncharacterized except that the mechanism that down-regulates the E_2-occupied ERα requires physical interaction between ERα and the transcriptional co-activator AIB1, whereas the mechanism, which down-regulates the ICI-182-780-occupied ERα, does not require AIB1 [16]. Unlike the estrogens and pure ERα antagonists, the mixed ERα agonist/antagonist, tamoxifen, induces up-regulation of ERα in cells [11–13,16]. Although the resistance of the tamoxifen-occupied ERα remains mechanistically unclear, it has been suggested that tamoxifen-induced up-regulation of ERα is due to resistance of the tamoxifen-occupied ERα to the proteasome [11–13,16].

Previously, we synthesized numerous hydroxy-*trans*-stilbenes, including 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), and studied their antioxidant and apoptotic activities [17–20]. Interestingly, 4,4′-DHS has been identified by other investigators as a specific ER ligand upon searching for natural and synthetic compounds that can bind to ER [21]. These investigators arbitrarily set the binding affinity of E_2 to ER of rat uterus at 100, and then calculated the relative binding affinities (RBA) of other compounds to the ER using data produced by in vitro competitive-binding assays [21]. It was determined that 4,4′-DHS was a specific ER ligand with an RBA of 0.281, which was much higher than the RBAs of bisphenol (RBA = 0.086) and Phenol Red (RBA = 0.001), two well-known ER agonists [21]. This information prompted us to investigate the effect of 4,4′-DHS on ERα in breast cancer cells, and found that 4,4′-DHS acted as an ERα antagonist and induced down-regulation of ERα.

2. Materials and methods

2.1. Materials

Tamoxifen, Na-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), leupeptin, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethylsulfonyl fluoride (PMSF), 17-β-estradiol (E_2), cycloheximide (CHX) and the mouse monoclonal antibodies against β-actin (A-5316), γ-tubulin (GTU-88) and PCNA (PC-10) were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA); MG132 *clasto*-lactacystin-β-lactone, epoxomycin, calpeptin and Z-VAD-FMK were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA); mouse monoclonal antibodies against ERα (F-10), ubiquitin (P4D1) and Hsp90 antibody (F-9) and rabbit polyclonal antibodies against ERα (MC-20), ERβ (H-150), c-Myc (9E10) and hTERT antibody (H-231) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); mouse monoclonal antibodies against topoisomerase 1 (#556597) and cyclin B1 (#554176) from PharMingen (San Diego, CA, USA) and rabbit polyclonal AIB1 antibody (S6505-08) from US Biological (Swampscott, MA, USA). ECL Western blot reagents were obtained from Amersham Pharmacia Biotechnologies Inc. (Piscataway, NJ, USA). The 3x-Vit-ERE-TATA-Luc plasmid, containing a luciferase gene under the control of ER [22] was kindly provided by Dr. Donald P. MacDonnell (Duke University Medical School, Durham, NC, USA). The compounds, 4-hydroxy-*trans*-stilbene (4-HS), 2,4-dihydroxy-*trans*-stilbene (2,4-DHS), 3,4-dihydroxy-*trans*-stilbene (3,4-DHS), 3,5-dihydroxy-*trans*-stilbene (3,5-DHS), 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), 3,4,4′-trihydroxy-*trans*-stilbene (3,4,4′-THS) and 4,4′-dimethoxy-*trans*-stilbene (4,4′-DMS) were synthesized as described [17,18], purified by column chromatography and re-crystallized. Mass spectroscopy (on a Bruker APEX II FT-MS spectrometer) and ^{1}H NMR (on a Bruker AM 400 NMR spectrometer) analyses confirmed the structures of the compounds. The purities of the compounds were determined to be 99.5, 99, 99.7, 98.2, 98.3, 99.2 and 99.8% for 4,4′-DHS, 2,4-DHS, 4-HS, 3,4,4′-THS,

3,4-DHS, 3,5-DHS and 4,4′-DMS, respectively, using a Hewlett-Packard 1100 HPLC system equipped with a diode array detector and a Phenomenex Nucleosil 5 μM C_{18} column (250 mm × 4.6 mm). Methanol–water (55:45) eluant was used in the HPLC analysis. All compounds were dissolved in dimethyl sulfoxide at a concentration of 100 mM and stored at 4 °C.

2.2. Cell cultures

The MCF-7 human breast cancer cell line was originally obtained from ATCC (Manassas, VA, USA), and was cultured in DMEM supplemented with fetal bovine serum (10%), penicillin (100 units/ml) and streptomycin (50 units/ml). The T47D human breast cancer cell line, provided by Dr. Devassis Chatterjee (Department of Clinical Pharmacology, Rhode Island Hospital, Providence, RI, USA), was cultured in RPMI 1640 medium supplemented with insulin (5 μg/ml), transferring (5 μg/ml), sodium selenite (5 ng/ml), fetal bovine serum (10%), penicillin (100 units/ml) and streptomycin (50 units/ml). All cell cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

2.3. Treatment of cells and Western blot analysis

Confluent cell cultures were treated with trypsin to detach the cells, which were subsequently washed and suspended in fresh culture medium, plated into culture dishes at approximately 50% confluency and cultured for 48 h. Then, the cells were left untreated or treated with 4,4′-DHS or other agents. Then, the cell culture medium was replaced with calcium- and magnesium-free phosphate-buffered saline (PBS), and the cells were scraped off the dish with a latex-rubber cell remover, transferred into a centrifuge tube and pelleted by gentle centrifugation. The cells were washed twice in PBS and then processed to yield whole cell extracts [23]. Aliquots (50 μg/sample) of the whole cell extracts were subjected to SDS-PAGE and Western blot analyses to detect ERα and other proteins using ECL reagents. The concentration of antibody for all Western blot analyses was 1 μg/ml.

2.4. Measurement of the transcriptional activity of ERα

MCF-7 cells were trypsinized, washed, re-suspended in DMEM free of Phenol Red and supplemented with 0.5% FBS, and plated into 24-well plates. The cells were cultured for 24 h to assume attachment to the substrate. The medium was removed, and the cells were washed three times with serum- and Phenol Red-free medium and cultured in serum- and Phenol Red-free medium for another 24 h. Subsequently, the cells were transfected for 4 h with 1 μg of 3x-Vit-ERE-TATA-luc plasmid DNA containing a luciferase reporter gene whose expression is dependent on ER [22]. The transfection was accomplished in the presence of the Fugene 6™ transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA). The transfected cells were cultured for 20 h in the absence (negative control) and presence of 10 nM E_2 (positive control) plus or minus various concentrations of 4,4′-DHS in Phenol Red-free medium containing 10% charcoal-stripped FBS. Subsequently, the amount of the luciferase in the cells was assayed using the Bright-Glo™ Luciferase Assay reagents (Promega Corporation, Madison, WI, USA). Typically, the presence of 10 nM E_2 for 24 h increased the amount of luciferase activity by approximately 3.6-fold.

2.5. RNA isolation and RT-PCR and Northern blot analyses

Total RNA was extracted from MCF-7 cells untreated and treated with 4,4′-DHS using TRIzol RNA extraction kit (Invitrogen). Any DNA contaminant in the RNA preparation was digested using RQ1 RNase free DNase (Promega). The RNA was then reverse transcribed and amplified using the One-tube and Two-enzyme Access RT-PCR System (Promega). Briefly, 125 ng of RNA were added to a tube containing reverse transcription/amplification buffer, dNTP mixture, gene specific primers, AMV reverse transcriptase and *Tfl* DNA polymerase. The reverse transcription reaction was carried out at 48 °C for 45 min followed by 23 cycles of the amplification reaction at 56 °C. The sequences of the primer sets used for the RT-PCR reaction are as follows: ERα forward primer, 5′-AGACATGAGAGCTGCCAACC-3′; ERα reverse primer, 5′-GCCAGGCACATTCTAGAAGG-3′; β-actin forward primer, 5′-ATCTGGCACCACACCTTCTACAAT-GAGCTGCG-3′; β-actin reverse primer, 5′-CGTCATACTCCTGC-TTGCTGATCCACATCTGC-3′. The β-actin mRNA was used to monitor the expression level of a housekeeping gene. Alternatively, 50 μg of total RNA were subjected to Northern blot analysis to detect ERα mRNA according to a previously described protocol [24]. GAPDH and p53 mRNAs were used as controls in the Northern blot analysis. ERα, GAPDH and p53 mRNAs were detected using cDNA probes that were radiolabeled with [α-^{32}P]dATP by the Random Priming reagents purchased from Stratagen.

3. Results

3.1. 4,4′-DHS inhibits transactivation of ERα by E_2

To study the effect of 4,4′-DHS on the transcriptional activity of ERα in our experiments, we used MCF-7 cells, which express ERα but not ERβ [25,26]. Briefly, estrogen-deprived MCF-7 cells were transfected with plasmid DNA containing a luciferase reporter gene whose expression is dependent on the transcriptional activity of ER [22]. The transfected cells were cultured for 20 h in the presence of E_2, 4,4′-DHS or E_2 + 4,4′-DHS and then the amount of luciferase activity in the cells was determined and used to represent the transcriptional activity of ERα [22]. In our experiments, we set the amount of luciferase activity in the cells treated for 24 h with 10 nM E_2 as the 100% base line transcriptional activity of ERα, and the effect of 4,4′-DHS on the expression level of the luciferase in the presence of 10 nM E_2 was determined. We consistently observed that 4,4′-DHS acted as a weak ERα antagonist in MCF-7 cells, because treatment of the cells for 24 h with 4,4′-DHS, at the concentration range of 2.5–20 μM, reduced the expression of the luciferase activity in a dose-dependent fashion (Fig. 1). We estimated that the IC_{50} value of 4,4′-DHS (the amount of 4,4′-DHS required to reduce the expression of the luciferase activity by 50% under the experimental conditions) was 4.8 ± 0.26 μM.

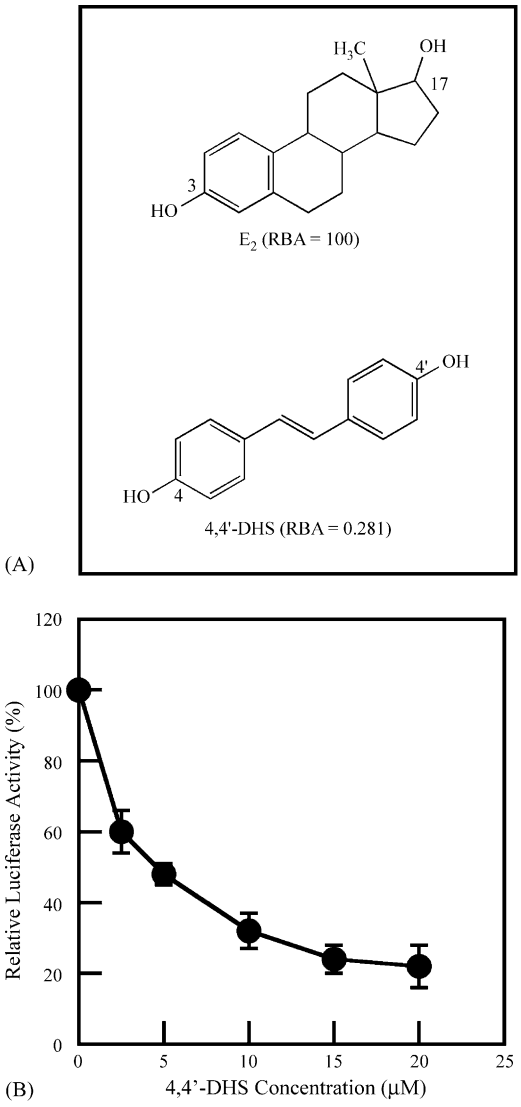


Fig. 1 – 4,4′-DHS inhibits ERα transactivation by E_2. (A) Structures and relative binding affinity (RBA) of E_2 and 4,4′-DHS to ER [21]. (B) 4,4′-DHS blocks, in a dose-dependent fashion, the ability of E_2 to stimulate ER-dependent expression of a luciferase reporter gene in MCF-7 cells (see Section 2). The amount of luciferase activity in the MCF-7 cells treated for 24 h with 10 nM E_2 alone was taken as the 100% base line transcriptional activity of ERα and the effect of 4,4′-DHS on the amount of luciferase ativity (i.e., ERα activity) in the presence of 10 nM of E_2 was compared to this base line.

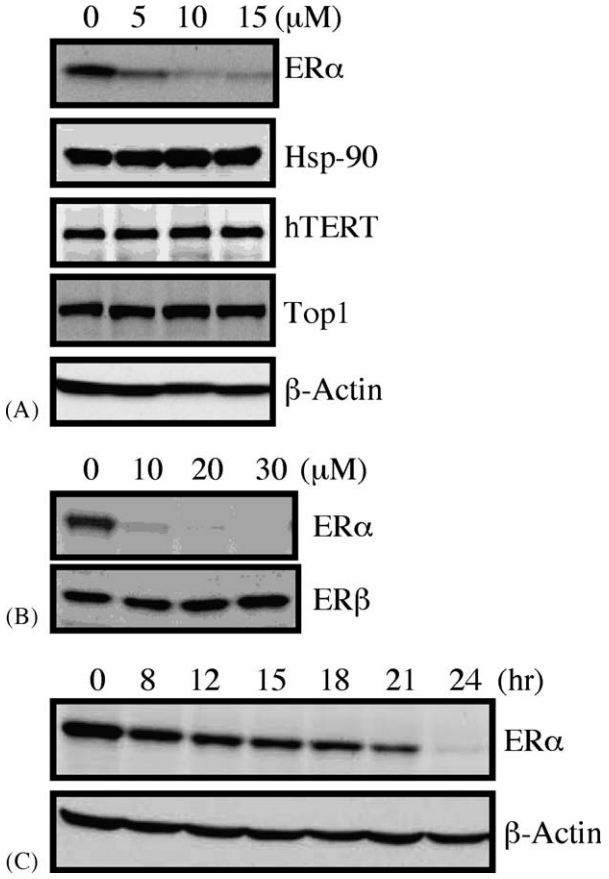


Fig. 2 – 4,4′-DHS induces down-regulation of ERα in MCF-7 and T47D cells. (A) MCF-7 cells were treated for 24 h with 0, 5, 10 and 15 μM of 4,4′-DHS. Then, whole cell protein extracts were analyzed by the Western blot technique to detect ERα and other proteins. (B) T47D cells were treated for 24 h with 0, 10, 20 and 30 μM of 4,4′-DHS. Then, whole cell protein extracts were analyzed by the Western blot technique to detect ERα and ERβ. (C) MCF-7 cells were treated with 15 μM 4,4′-DHS for the indicated lengths of time and whole cell protein extracts were analyzed by the Western blot technique to detect ERα.

3.2. *4,4′-DHS induces selective down-regulation of ERα*

We observed that treatment of MCF-7 cells for 24 h with 5–15 μM of 4,4′-DHS induced down-regulation of ERα in a dose-dependent fashion, but had no significant effect on the levels of other proteins, including the nuclear proteins, Hsp90, hTERT and Top1, and the cytoplasmic protein β-actin (Fig. 2A). Subsequently, we observed that treatment of T47D breast cancer cells, which express both ERα and ERβ, for 24 h with 10 μM of 4,4′-DHS resulted in nearly complete down-regulation of ERα, whereas a 24 h treatment of the cells with up to 30 μM of 4,4′-DHS virtually had no effect on the level of ERβ (Fig. 2B). These observations indicated that 4,4′-DHS induced selective down-regulation of ERα in T47D cells.

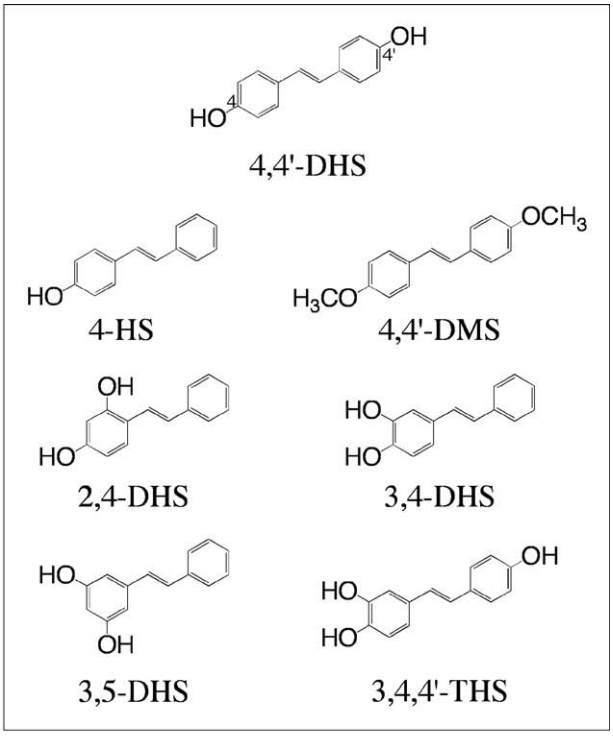
Since treatment of MCF-7 cells with the high affinity pure ERα antagonist, ICI-182-780, for 1 h was sufficient to induce significant down-regulation of ERα [9–14], we investigated whether down-regulation of ERα would also occur rapidly in these cells following treatment with 4,4′-DHS. The results showed that treatment of the cells with 15 μM of 4,4′-DHS induced slow and gradual down-regulation of ERα over a period of 21 h (Fig. 2C). Therefore, 4,4′-DHS was a slow down-regulator of ERα.

3.3. *The positions of the hydroxyl groups are essential for the ability of 4,4′-DHS to induce down-regulation of ERα*

It was predicted, based on the crystal structure of the ligand-bound LBD of human ERα [27–29], that both 4-OH and 4′-OH should be essential for 4,4′-DHS to stably bind to ERα [21]. To investigate the importance of the hydroxyl groups for the ability of 4,4′-DHS to induce down-regulation of ERα, we compared the ability of 4-HS, 2,4-DHS, 3,4-DHS, 3,5-DHS, 3,4,4′-THS and 4,4′-DMS (Fig. 3) with that of 4,4′-DHS to induce down-regulation of ERα in MCF-7 cells. The results showed that 2,4-DHS, 3,4-DHS, 3,5-DHS and 3,4,4′-THS virtually had no effect on the amount of ERα and 4-HS was less effective than 4,4′-DHS, whereas 4,4′-DMS lacked the ability to induce down-regulation ERα (Fig. 3). These results demonstrated that the presence of both 4-OH and 4′-OH groups was required for 4,4′-DHS to induce down-regulation of ERα.

3.4. *Induction of down-regulation of ERα by 4,4′-DHS requires a mechanism at the post-transcriptional level*

To assess whether 4,4′-DHS-induced down-regulation of ERα was the result of reduced expression of ERα mRNA, we compared the levels of ERα mRNA in MCF-7 cells untreated and treated for 24 h with 15 μM of 4,4′-DHS. This was accomplished by RT-PCR and Northern blot analyses of the RNA isolated from the cells. The results of both RT-PCR (Fig. 4A) and Northern blot analyses (Fig. 4B) showed that there was no significant difference between the expression levels of ERα mRNA from untreated and treated cells, and hence,



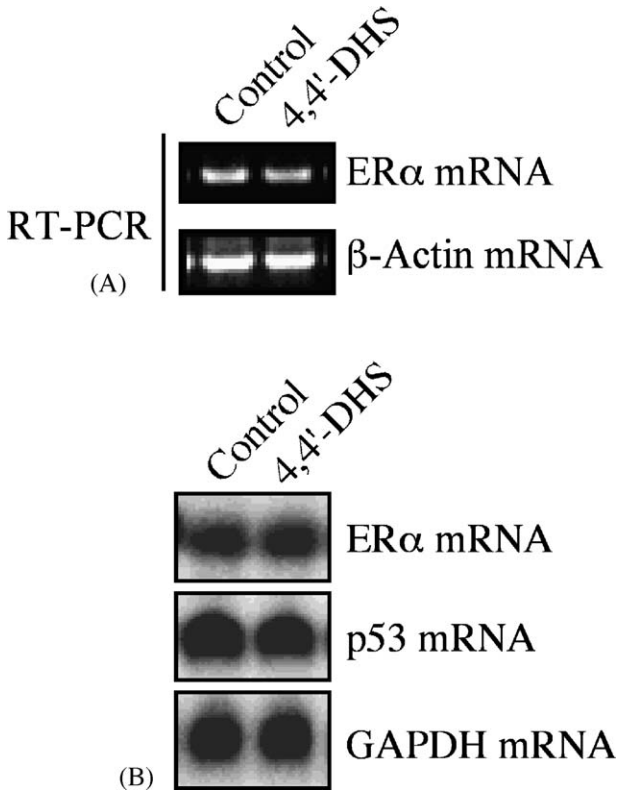


Fig. 3 – (A and B) The 4-OH and 4′-OH groups are essential for the activity of 4,4′-DHS. The structure of 4,4′-DHS and its analogues are shown. MCF-7 cells were left untreated or treated for 24 h with 20 μM of 2,4-DHS or 3,4-DHS or 3,5-DHS or 3,4,4′-THS. Alternatively, MCF-7 cells were treated for 24 h with 4,4′-DHS, 4-HS and 4,4′-DMS at the indicated concentrations. Whole cell protein extracts were analyzed by the Western blot to detect ERα.

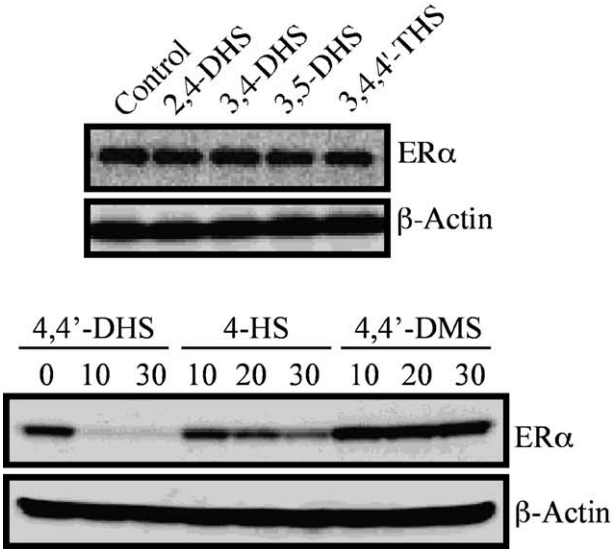


Fig. 4 – 4,4′-DHS induces down-regulation of ERα at a post-transcriptional level. MCF-7 cells were left untreated (control) or treated for 24 h with 15 μM 4,4′-DHS and total RNAs were isolated and subjected to: (A) RT-PCR and (B) Northern blot analyses to detect alteration in the ERα mRNA level. β-actin, GAPDH and p53 mRNAs were used as controls.

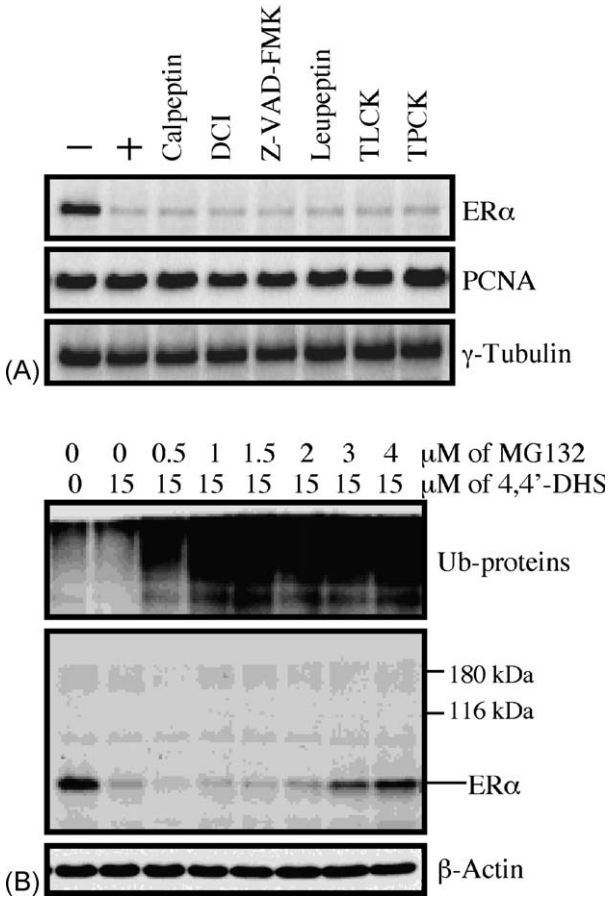


Fig. 5 – MG132 blocks the ability of 4,4′-DHS to induce ERα down-regulation. (A) MCF-7 cells were left untreated or treated with 15 μM of 4,4′-DHS for 24 h in the absence or presence of MG132 at the indicated concentrations and whole cell protein extracts were analyzed by Western blot technique to detect ubiquitinated proteins (Ub-proteins), ERα and β-actin. (B) MCF-7 cells were left untreated (–) or treated (+) for 24 h with 15 μM 4,4′-DHS. Alternatively, the cells were treated for 24 h with 15 μM 4,4′-DHS in the presence of 100 μM of calpeptin, 50 μM of 3,4-dichloroisocoumarin (DCI), 100 μM of Z-VAD-FMK, 50 μM of leupeptin, 50 μM of TLCK or 50 μM of TPCK. Subsequently, aliquots of whole cell protein extracts were subjected to Western blot analysis to detect ERα and other proteins.

suggested that 4,4′-DHS-induced down-regulation of ERα was the result of an event at the post-transcriptional rather than the transcriptional level.

3.5. *MG132, but not other proteasome inhibitor, can block 4,4′-DHS-induced down-regulation of ERα*

To determine whether 4,4′-DHS would induce degradation of ERα by a protease, we first investigated the effect of various protease inhibitors as well as the proteasome inhibitor, MG132, on the ability of 4,4′-DHS to induce down-regulation of ERα in MCF-7 cells. The results showed that calpeptin (a relatively specific calpain inhibitor), TLCK (a general inhibitor of trypsin-like serine proteases), TPCK (a general inhibitor of chymotrypsin-like serine proteases), leupeptin (an inhibitor of cystein proteases), 3,4-dichloroisocoumarin (a general inhibitor of serine proteases) and Z-VAD-FMK (a pan-caspase inhibitor) had no effect on the ability of 4,4′-DHS to induce down-regulation of ERα (Fig. 5A). In contrast, treatment of the cells with concentrations of ≥3 μM of MG132 significantly prevented 4,4′-DHS-induced down-regulation of ERα (Fig. 5B). Interestingly, treatment of the cells with approximately 1 μM of MG132 was sufficient to induce maximal accumulation of ubiquitinated proteins, i.e., maximal inhibition of the proteasome (Fig. 5B). Thus, it appeared that the MG132 amount required to prevent 4,4′-DHS from inducing down-regulation of ERα exceeded the amount required to maximally inhibit the proteasome in MCF-7 cells. Therefore, it was unclear whether MG132-mediated inhibition of the proteasome was responsible for preventing 4,4′-DHS-induced down-regulation of ERα. To clarify this issue, we treated MCF-7 cells for 24 h with 15 μM of 4,4′-DHS in the absence and presence of the proteasome inhibitors, *clasto*-lactacystin-β-lactone and epoxomycin [30,31] and then determined proteasome inhibition and the level of ERα. The results showed that both *clasto*-lactacystin-β-lactone and epoxomycin effectively inhibited the proteasome in the cells as shown by the accumulation of a large quantity of ubiquitinated proteins and up-regulation of the well-charac-

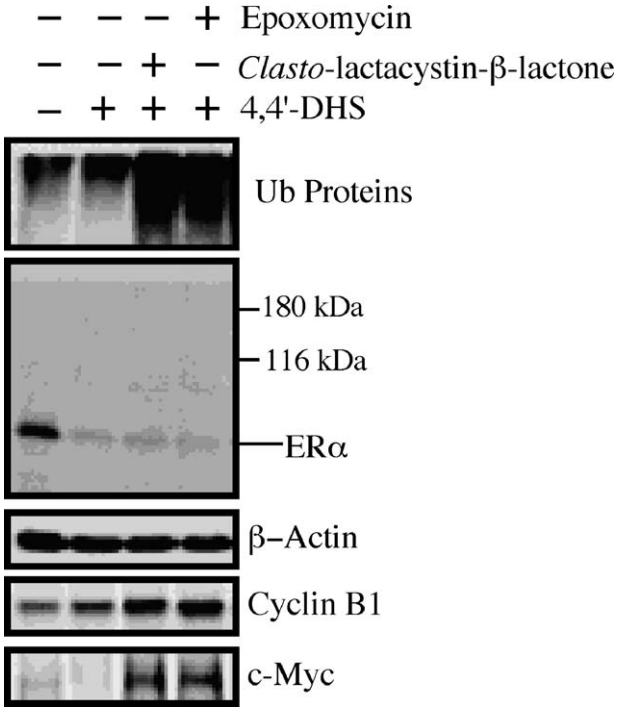


Fig. 6 – *Clasto*-lactacystin-β-lactone and epoxomycin inhibits the proteasome but do no block 4,4′-DHS-induced ERα down-regulation. MCF-7 cells were left untreated or treated with 15 μM of 4,4′-DHS for 24 h in the absence or presence of 15 μM *clasto*-lactacystin-β-lactone or 50 nM epoxomycin and whole cell protein extracts were subjected to Western blot analyses for the presence of ubiquitinated proteins (Ub-proteins), ERα, β-actin, cyclin Bl and c-Myc.

terized proteasome-targeted proteins, cyclin B1 [32] and c-Myc [33] in the cells (Fig. 6). However, neither *clasto*-lactacystin-β-lactone nor epoxomycin prevented 4,4′-DHS-induced down-regulation of ERα in MCF-7 cells (Fig. 6). These results demonstrated that 4,4′-DHS-induced down-regulation of ERα was proteasome-independent, and suggested that the ability of MG132, at concentrations ≥3 μM, to prevent 4,4′-DHS-induced down-regulation of ERα in MCF-7 cells was unrelated to its ability to inhibit the proteasome.

4. Discussion

4.1. 4,4′-DHS induces proteasome-independent down-regulation of ERα

The proteasome inhibitors, *clasto*-lactacystin-β-lactone, epoxomycin and MG132, have been widely used to treat cells in order to identify proteasome-dependent cellular activities [34]. *Clasto*-lactacystin-β-lactone and epoxomycin, are the most specific inhibitors of the proteasome known to date [30,31]. *Clasto*-lactacystin-β-lactone may interact with other target(s) in addition to the proteasome in cells, but the proteasome is currently the only known cellular target of epoxomycin [31]. Nevertheless, it appears that if a cellular activity is sensitive to *clasto*-lactacystin-β-lactone and epoxomycin, then it is reasonable to deduce that the activity is proteasome-dependent [30,31]. Further, MG132 is a less specific proteasome inhibitor than *clasto*-lactacystin-β-lactone and epoxomycin [30,31], and therefore, any effect of MG132 on a cellular activity does not necessarily suggest a dependence of the activity on the proteasome, unless the effect of MG132 can be confirmed by other proteasome inhibitors, such as *clasto*-lactacystin-β-lactone and epoxomycin.

It is widely believed that estrogens and several pure ERα antagonists, such as ICI-182-780 and GW5638, induce proteasome-dependent down-regulation of ERα in cells, mainly because treatment of various types of cells, including MCF-7 cells, with either MG132 or *clasto*-lactacystin-β-lactone prevents ER ligands from inducing down-regulation of ERα [9–14]. This notion is further supported by studies showing that E_2 induces ubiquitination of ERα in MCF-7 and 293 cells [13,15], and that ERα can be ubiquitinated and consequently degraded by the proteasome in in vitro assays [8]. In this study, we observed that 4,4′-DHS acted as an ERα antagonist and induced slow and selective down-regulation of ERα in MCF-7 and T47D breast cancer cells (Figs. 1 and 2). Our observations indicate that 4,4′-DHS-induced down-regulation of ERα is proteasome-independent. First, both *clasto*-lactacystin-β-lactone and epoxomycin, at the concentrations we used, effectively inhibited the proteasome, and consequently resulted in extensive accumulation of ubiquitinated proteins and up-regulation of the well-characterized proteasome targeted proteins, cyclin B1 [32] and c-Myc [33], in MCF-7 cells, but the presence of either *clasto*-lactacystin-β-lactone or epoxomycin prevented 4,4′-DHS from inducing down-regulation of ERα (Fig. 6). Second, the presence of 1 μM of MG132 was sufficient to induce maximal accumulation of ubiquitinated proteins, i.e., maximal inhibition of the proteasome in MCF-7 cells, but it did not prevent 4,4′-DHS from inducing down-regulation of ERα in the cells under our experimental conditions (Fig. 5B). Rather, the presence of MG132 concentrations of ≥3 μM was required to effectively prevent 4,4′-DHS-induced down-regulation of ERα (Fig. 5B). Therefore, the amount of MG132 required to prevent 4,4′-DHS-induced down-regulation of ERα was much larger than the amount required to maximally inhibit the proteasome in MCF-7 cells (Fig. 5B). Third, had 4,4′-DHS induced proteasome-dependent down-regulation of ERα, a significant number of ERα molecules should have been ubiquitinated in the 4,4′-DHS-treated cells, and hence, treatment of the cells with 4,4′-DHS, in the presence of MG132, *clasto*-lactacystin-β-lactone or epoxomycin, should have resulted in accumulation of ubiquitinated ERα molecules, which would have appeared as additional high molecular bands upon ERα-specific Western blot analysis. However, the Western blot analysis of the MCF-7 cells treated with 4,4′-DHS in the presence of MG132, *clasto*-lactacystin-β-lactone or epoxomycin showed the ERα band and no bands of high molecular weights corresponding to ubiquitinated ERα (Figs. 5b and 6). Therefore, we conclude that 4,4′-DHS induces proteasome-independent down-regulation of ERα.

We hypothesized that MG132, at concentrations of ≥3 μM, was capable of interacting with and subsequently inhibiting a non-proteasomal factor essential for 4,4′-DHS to induce down-regulation of ERα. It is plausible that this MG132-sensitive factor is a protease that degrades ERα in the presence of 4,4′-DHS. If so, then this putative protease is insensitive to the general inhibitors of calpain, trypsin-like serine proteases, chymotrypsin-like serine proteases, serine proteases, certain cystein proteases and caspases (Fig. 5A), as well as the proteasome inhibitors, *clasto*-lactacystin-β-lactone and epoxomycin (Fig. 6). Identification and characterization of this putative protease will lead to development of a new process that down-regulates ERα.

It has come to our attention that 5–10 μM, or even higher concentrations, of MG132 are commonly used to treat various cell types for 24–48 h in studies to identify proteasome-dependent cellular activities. Furthermore, a large number of these studies do not use additional proteasome inhibitors to confirm the effect of MG132 on the cells. During the course of this study, we investigated the dose-dependent effect of MG132 on the proteasome in diverse types of cultured cells and found that a 12–20 h treatment with 1–1.5 μM of MG132 was sufficient to induce maximal inhibition of the proteasome (data not shown). Therefore, we suggest that caution should be taken when interpreting MG132-altered cellular activities as proteasome-dependent activities.

4.2. The structure-associated activity of 4,4′-DHS

We demonstrated that both 4-OH and 4′-OH groups were important for the ability of 4,4′-DHS to induce down-regulation of ERα in MCF-7 cells (Fig. 3). Based on the information of the crystal structure of the LBD of human ERα [27–29], it was suggested that binding of 4,4′-DHS to ERα depends on the ability of 4-OH and 4′-OH to form hydrogen bonding with Glu353 and His524, respectively, in the ligand-binding cavity of the LBD, and that 4-HS binds much less stably than 4,4′-DHS to ERα [21]. Thus, our finding showing that 4-HS was less able than 4,4′-DHS to induce down-regulation of ERα (Fig. 3) suggests that 4-OH and

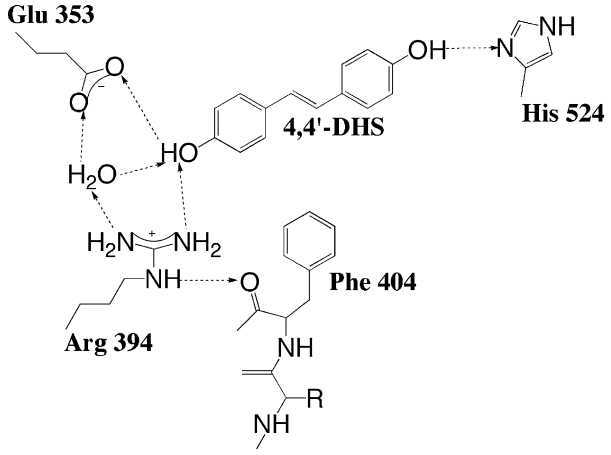


Fig. 7 – Hydrogen bonding between 4,4′-DHS and ERα. The hydrogen bonds (dashed lines with arrow heads) between the 4-OH of 4,4′-DHS and Glu353 and between the 4′-OH of 4,4′-DHS and His350 in the ligand-binding domain (LBD) of ERα are predicted [21].

4′-OH enables 4,4′-DHS to stably bind to and induce down-regulation of ERα. The importance of 4-OH and 4′-OH for the activity of 4,4′-DHS is further underscored by the fact that substituting the OH groups with methoxyl groups in 4,4′-DHS yielded 4,4′-DMS that failed to induce down-regulation of ERα (Fig. 3). Interestingly, 3,4,4′-THS also failed to induce down-regulation of ERα (Fig. 4). Hence, the presence of an *ortho*-OH to 4-OH severely negate the activity of 4,4′-DHS.

It has been suggested that the presence of hydrophobic groups on the *trans* double bond of a compound like 4,4′-DHS would allow it to form hydrophobic interactions with residues in the LBD of ERα and thus binds more stably to ERα (Fig. 7, ref. [21]). It is also possible that addition of hydrophobic moieties to the *trans* double bond of 4,4′-DHS increases its binding affinity to ERα [21]. Therefore, it will be interesting to investigate whether addition of certain hydrophobic moieties to the *trans* double bond of 4,4′-DHS would yield derivatives with higher binding affinity and higher ability to induce down-regulation of ERα. However, it is already known that addition of simple alkyl moieties to the *trans* double bond of 4,4′-DHS transforms it into potent estrogens, such as diethylstilbestrol [34]. Nevertheless, we suggest that the structure of 4,4′-DHS provides a useful basic scaffold for the development of ERα ligands, which can induce down-regulation of ERα independent of the proteasome.

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