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ORIGINAL LABORATORY INVESTIGATION

Complete elimination of estrogen receptor α by PROTAC estrogen receptor α degrader ERD-148 in breast cancer cells

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Abstract

Purpose Estrogen Receptor α (ERα) is a well-established therapeutic target for Estrogen Receptor (ER)-positive breast cancers. Both Selective Estrogen Receptor Degraders (SERD) and PROTAC ER degraders are synthetic compounds suppressing the ER activity through the degradation of ER. However, the differences between SERD and PROTAC ER degraders are far from clear.

Methods The effect of PROTAC ER degrader ERD-148 and SERD fulvestrant on protein degradation was evaluated by western blot analysis. The cell proliferation was tested by WST-8 assays and the gene expressions were assessed by gene microarray and real-time RT-PCR analysis after the compound treatment.

Results ERD-148 is a potent and selective PROTAC ERα degrader. It degrades not only unphosphorylated ERα but also the phosphorylated ERα in the cells. In contrast, the SERD fulvestrant showed much-reduced degradation potency on the phosphorylated ERα. The more complete degradation of ERα by ERD-148 translates into a greater maximum cell growth inhibition. However, ERD-148 and fulvestrant share a similar gene regulation profile except for the variation of regulation potency. Further studies indicate that ERD-148 degrades the ERα in fulvestrant-resistant cells.

Conclusion PROTAC ER degrader has a different mechanism of action compared to SERD which may be used in treating fulvestrant-resistant cancers.

Keywords Fulvestrant · Estrogen receptor α · PROTAC ER degrader · MCF7 · Breast cancer · Phosphorylated ERα

Abbreviations

ER	Estrogen receptor
ERD	Estrogen receptor degrader
FITC	Fluorescein-5-isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Imax	Maximum inhibition
PCR	Polymerase chain reaction
PI	Propidium iodide
PROTAC	Proteolysis-targeting chimeras
PVDF	Polyvinylidene difluoride
RIPA buffer	Radioimmunoprecipitation assay buffer
SERD	Selective estrogen receptor degraders
SERM	Selective estrogen receptor modulators

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Introduction

Estrogen receptors mediate estrogen's biological effects, and more than 70% of breast cancers are Estrogen Receptor (ER) positive [1–3]. Two isoforms of estrogen receptors, ERα and ERβ, were identified in humans [4, 5]. The ERα is considered the major mediator that transduces estrogen signaling in the female reproductive tract and mammary glands [5, 6].

The binding of estrogen to the estrogen receptors induces ER phosphorylation and activates the estrogen receptor[2, 7, 8]. The phosphorylated estrogen receptors were then translocated to the nucleus, where they interact with the ER binding elements and other transacting factors to regulate the target gene expression[2, 7, 8].

Selective Estrogen Receptor Degraders (SERD) are small molecules that bind to the ER and induce misfolding of the estrogen receptors, which results in proteasome-dependent ER protein degradation[9–11]. Currently, the fulvestrant is the only FDA-approved SERD that has been used for treating advanced ER-positive breast cancer along with standard endocrine therapies in postmenopausal patients [10, 12].

Other SERDs, such as GDC0810 and AZD9496, are also developed and under clinical trials[11, 13]. SERD has been found to overcome the drug resistance of Selective Estrogen Receptor Modulators (SERM) and have a better suppression of the activity of estrogen receptors[9, 11].

Proteolysis-targeting chimeras (PROTAC) ER degraders are a different kind of ER degraders compared to SERD[14–24]. They utilized heterobifunctional small molecules containing ER ligands and ligands for an E3 ligase system [14, 15, 17]. These two ligands, when tethered together by a chemical linker, will hijack the cellular E3 ubiquitination ligase systems and cause the selected degradation of ERα protein[14, 15, 17, 18, 25]. To date, many PROTAC ER degraders that can efficiently degrade the ER both in vitro and in vivo have been reported[14, 15, 18–26].

Although both PROTAC ER degrader and SERD degrade the ER, the differences between these two kinds of ER degraders are still largely unknown [18–24]. In this study, the PROTAC ER degrader ERD-148, SERD fulvestrant, and SERM raloxifene were used as example compounds. Their effects on breast cancer cells were compared.

Methods

Compounds and chemicals

All the chemicals were purchased from Sigma-Aldrich, Inc. St. Louis, MO, Selleck Inc., Houston, TX or synthesized in the lab with more than 95% purity.

Cell culture

ER expression human breast cancer cell lines MCF7 (ATCC HTB-22) and T47D (ATCC HTB-133) were purchased from the American Type Culture Collection (ATCC), Manassas, VA. To create the fulvestrant-resistant MCF7 cell line, the MCF7 cells were cultured and maintained in a culture medium containing 100 nM Fulvestrant for more than three months before use.

Antibodies

The anti-estrogen receptor α antibody (catalog No. ab108398) and anti-estrogen receptor β antibody (ab3576) were purchased from Abcam Inc. Anti-phospho-estrogen receptor α (Ser118) (catalog No. SAB4300054) was purchased from Sigma-Aldrich, Inc., St. Louis, MO. IRDye® 800CW goat anti-rabbit IgG and IRDye® 680RD donkey anti-mouse IgG secondary antibodies were purchased from LI-COR Biosciences, Lincoln, NE, USA. All the other antibodies were purchased from Santa Cruz Biotechnology, Inc., Dallas, TX.

Western blot analysis

Western blot analysis was performed as described previously[15, 27]. The blots were scanned and quantified using the Odyssey® DLx Imaging System from LI-COR Biosciences, Lincoln, NE, USA. The relative quantities were calculated after normalized to the corresponding GAPDH loading controls.

DNA microarray and bioinformatics analysis

The total RNA samples were amplified using the GeneChip WT Plus kit from Affymetrix and hybridized with a Human Gene ST 2.1 plate following the protocol from Fisher Scientific, Pittsburgh, PA. The gene chip was scanned by Axon GenePix 4000B microarray scanner, with NimbleScan (version 2.5) software for the image analysis which converts the image signal to a digital signal. The expression values for each gene were calculated using a robust multi-array average (RMA) model fitting developed by Irizarry et al. [28]. The oligo package of Bioconductor 6 implemented in the R statistical environment was used for further microarray analysis[28, 29].

Quantitative RT-PCR analysis (qRT-PCR)

One-step real-time reverse transcriptase PCR was conducted using a QuantStudio™ 7 Flex machine from Thermo Fisher Scientific™. The gene expression was evaluated against the vehicle-treated controls with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA used as the internal control. All the reagents were purchased from Applied Biosystems (Waltham, MA, USA).

Annexin V/propidium iodide staining

Measurement of cell death was conducted with dead cell apoptosis kits with Annexin V (Catalog No. V13242) from Thermo Fisher Scientific, Inc., following the instruction manual from the company.

Cell cycle analysis

The MCF7 cells were treated with indicated compounds or vehicle control for 48 h. The cells were then washed with PBS and fixed with cold 70% ethanol for 30 min. After washing with 1xPBS two times to remove the ethanol residue, the cells were digested with RNase for 1 h and stained with propidium Iodide overnight at 4 °C. The stained cells

were then analyzed by Attune NxT acoustic focusing cytometer (Thermo Fisher Scientific, Inc., Waltham, MA). Single-cell populations were gated, and the mitosis stage of the cells was determined by the DNA contents in the cells using the Flowjo software V10 from BD Bioscience, Inc., Ashland, OR.

Cell growth assay

MCF7 or T47D cells were seeded on 96-well plates and treated with indicated doses of compounds, respectively. Four days after the compound treatment, the growth of the cells was evaluated by colorimetric WST-8 assay following the instructions of the manufacturer, Cayman Chemical, Ann Arbor, MI.

Statistical analysis

The differences between the treated samples and control samples were evaluated by t-test. A value of $p < 0.05$ was used as a criterion and marked with * for statistical significance in comparisons between treated samples and control samples.

Results

Compound ERD-148 is a PROTAC estrogen receptor α degrader

Linking the raloxifene and VHL ligand through a 7-carbon linker, a compound was generated and was named ERD-148 (Fig. 1a) [15, 25]. To confirm the ERD-148 is a PROTAC ER degrader, MCF7 and T47D cells were pretreated with 1 μM ER inhibitor raloxifene, VHL ligand, proteasome inhibitor carfilzomib or the vehicle controls, respectively, in complete medium for 10 min. Then 30 nM ERD-148, 30 nM Fulvestrant, or the vehicle DMSO were added, respectively, as indicated and incubated for 4 h. The total proteins were then analyzed by western blotting analysis. The result shown in Fig. 1c indicates that the treatment of ERD-148 alone leads to a more than 90% decrease of ERα in the cell compared to the vehicle-treated control (lane leftmost). Treatment of Fulvestrant alone leads to ~ 70% decrease in ERα proteins in the cell. Pretreatment of the cells with the ER inhibitor raloxifene or proteasome inhibitor carfilzomib abolished the reduction of ERα by both ERD-148 and fulvestrant treatment. In addition, the ERD-148-induced ERα degradation was abolished by pretreating the cells with VHL ligand. In contrast, the ERα reduction caused by fulvestrant treatment is essentially unchanged by pretreatment of the VHL ligand (Fig. 1b and c). This result confirmed that the ERD-148 is a PROTAC ER degrader and the Fulvestrant is a SERD-type degrader.

Compound ERD-148 achieves more complete degradation of ERα than fulvestrant

It is interesting to see if SERD and PROTAC ER degraders have any differences in terms of degrading the ER. Since fulvestrant is a very potent and the first FDA-approved SERD ERα degrader, it was chosen as an example of SERDs and compared with the ERD-148, one of the potent PROTAC ER degraders. We treated the MCF7 breast cancer cells with either fulvestrant or ERD-148 at doses ranging from 0 to 1 μM for 4 h and analyzed the ERα level in the cell by western blot analysis. As shown in Fig. 1d, compound ERD-148 achieves a DC50 value of 0.3 nM and a > 90% maximum ERα degradation at ~ 30 nM with a 4-h treatment in the MCF7 cells. In contrast, the SERD fulvestrant achieves a DC50 value of 3 nM and a > 77% maximum ERα degradation at ~ 30 nM with a 4-h treatment in the MCF7 cells (Fig. 1e). In addition, at concentrations higher than 300 nM, less ERα degradation than at lower concentrations was observed in ERD-148-treated samples, a typical "hook effect" that has been reported in PROTAC degraders (Fig. 1d). In contrast, no "hook effect" was observed in fulvestrant-treated samples. At high concentrations of fulvestrant treatment, the ERα remains essentially the same as in lower concentration treatments after it reaches the maximum degradation at 10 nM concentrations (Fig. 1e). This result suggests that although both fulvestrant and ERD-148 treatments reduced the ERα level in the MCF7 cells, only the PROTAC ERα ERD-148 showed a "hook effect" and it achieved more complete degradation of ERα than the SERD fulvestrant.

Compound ERD-148 and fulvestrant have similar kinetics of ERα degradation

According to the dose response of MCF7 to the compound ERD-148 and fulvestrant treatment, at a concentration of 30 nM, both compounds can achieve maximum degradation of ERα in the cell. Thus, to compare the kinetics of these ERα degraders, the MCF7 and T47D breast cancer cells were treated with compound ERD-148 and fulvestrant at 30 nM, respectively. We treated the MCF7 cells and extracted the total proteins at different intervals after the compound treatment. As shown in Fig. 1f, the ERα protein level was only slightly reduced after 1 h of compound treatment. The maximum ERα degradation is achieved around 3 h after compound treatment and keeps steady until 24 h after treatment for both ERD-148 and fulvestrant-treated MCF7 cells. In T47D cells, more than 50% reduction of the ERα level in the cell was observed 1 h after the compound

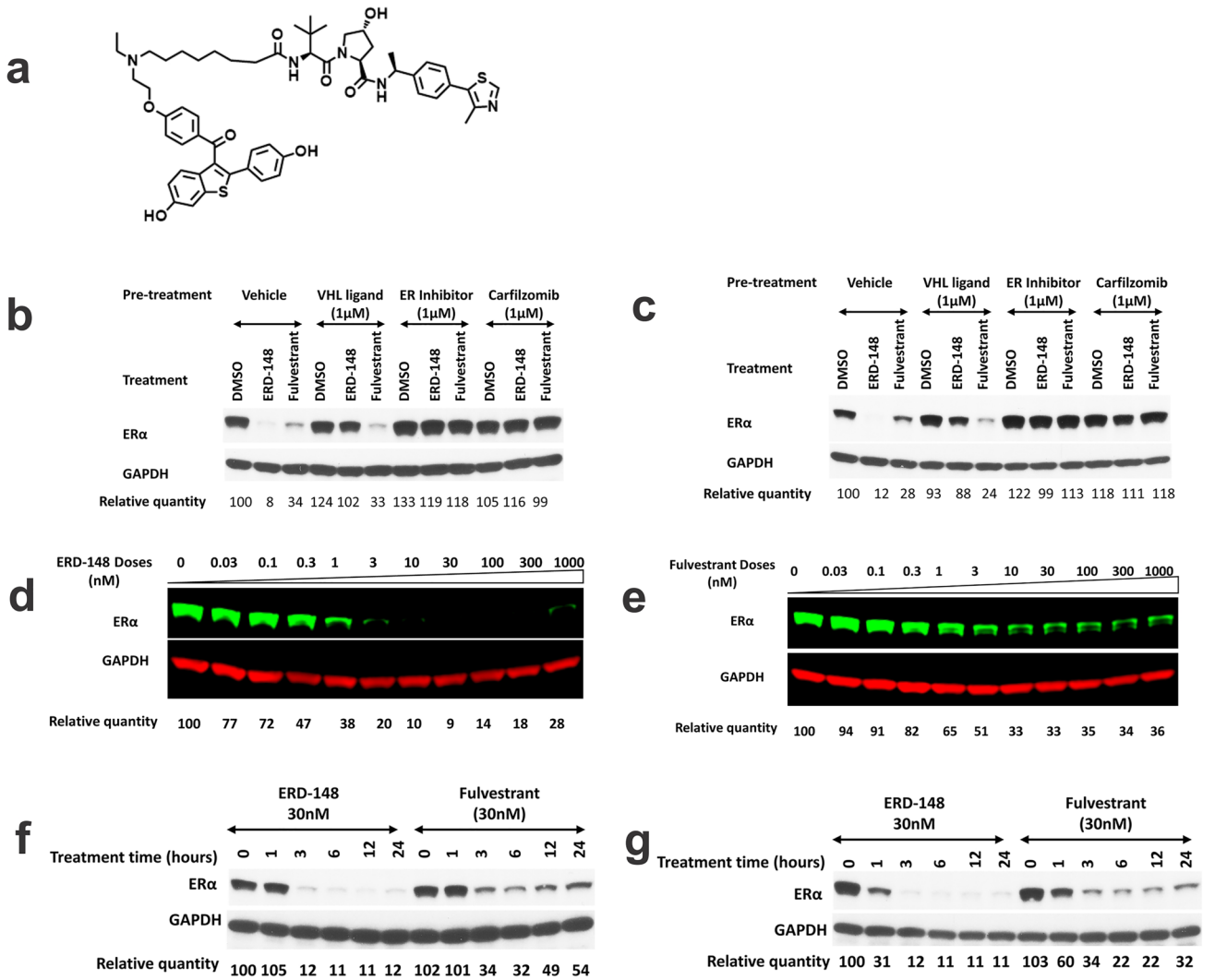

Fig. 1 Dose responses and kinetics of PROTAC ERα degrader ERD-148 and SERD fulvestrant. **a** Structure of compound ERD-148. **b** MCF7 and T47D cells were pretreated with 1 μM ER inhibitor raloxifene, VHL ligand, proteasome inhibitor carfilzomib, and the vehicle controls, respectively, in a complete medium for 10 min. Then 30 nM ERD-148, 30 nM fulvestrant, and the vehicle DMSO were added, respectively, as indicated, and incubated for 4 h. The total proteins were then extracted in RIPA buffer and analyzed by western blotting analysis. **c** The T47D cells were treated the same way as previously in MCF7 cell (**b**). The ERα level after the treatment was evaluated by western blotting analysis. **d** Dose response of MCF7 cells to ERD-148 after 4 h of treatment as evaluated by western blotting analysis for ERα. **e** Dose response of MCF7 cells to fulvestrant after 4 h treatment as evaluated by western blotting analysis for ERα. **f** Kinetics of ERα degradation evaluated by western blotting analysis after 30 nM ERD-148 or 30 nM fulvestrant treatment in MCF7 cells. **g** Kinetics of ERα degradation evaluated by western blotting analysis after 30 nM ERD-148 or 30 nM fulvestrant treatment in T47D cells

treatment (Fig. 1g). The maximum degradation of ERα was achieved about 3 h after the compound treatment and kept steady until the end of the test (Fig. 1g). Taken together, these results suggest that ERD-148 degrader and fulvestrant have similar kinetics of ERα degradation.

Compound ERD-148 degrades phosphorylated estrogen receptors

The MCF7 cells were treated with 100 pM 17β-Estradiol for the indicated time interval. The total proteins were then extracted in RIPA buffer containing phosphatases inhibitor (phos-STOP from Roche) and analyzed by western blot analysis with antibodies specific for phosphorylated ERα and total ERα. The result confirmed that MCF7 cells contain both phosphorylated and unphosphorylated ERα forms (Fig. 2a and b). 17β-Estradiol treatment increases both phosphorylated ERα with a peak at 0.5 h after treatment (Fig. 2a) and the total ERα with a peak at 12 h after treatment (Fig. 2b).

To investigate if the phosphorylation modification of ERα affects the ERD-148 and fulvestrant induced ER

degradation, the MCF7 were treated with the indicated dose of ERD-148 and fulvestrant, respectively. Four hours after the treatment, the total proteins were extracted and analyzed by western blot analysis with antibodies specific for Ser^{-118} phosphorylated ERα. The result shown in Fig. 2c indicates that compound ERD-148 decreases the phosphorylated ERα in the cell with a DC50 value of 0.3 nM and a > 90% maximum degradation at 10 nM. In contrast, less than 10% of Ser^{-118} phosphorylated ERα was reduced by fulvestrant treatment compared to vehicle-treated control (Fig. 2d).

To further test if the phosphorylation of ERα affects its degradation induced by other SERDs, the MCF7 cells were treated with GDC0810 and AZD9496 at indicated doses for 4 h. The level of total ERα and Ser^{-118} phosphorylated ERα in the total cell lysate was then evaluated by western blot analysis. The result confirmed that GDC0810 and AZD9496 treatment reduced the total ERα in the cell and achieved maximum degradation of ERα 51% at 1uM for GDC0810 and 42% at 100 nM for AZD9496, respectively (Fig. 3a and c). In contrast, only 15% and 16% of Ser^{-118} phosphorylated ERα were reduced by GDC0810 and AZD9496 treatments, respectively (Fig. 3b and d).

ERD-148 degrades ERα in fulvestrant-resistant MCF7

To confirm the different degradation mechanisms between PROTAC ER degrader and SERD, fulvestrant-resistant MCF7 cells were treated with ERD-148 or fulvestrant for 4 h. As indicated in Fig. 4, the MCF7 cells after long-term culturing are resistant to fulvestrant treatment. Only a minimal reduction of ERα at a high dose of fulvestrant treatment was observed. Interestingly, the ERα level in the MCF7-resistant cells is further reduced by ERD-148 treatment (Fig. 4a and b) and the fulvestrant-resistant MCF7 cell growth was also suppressed

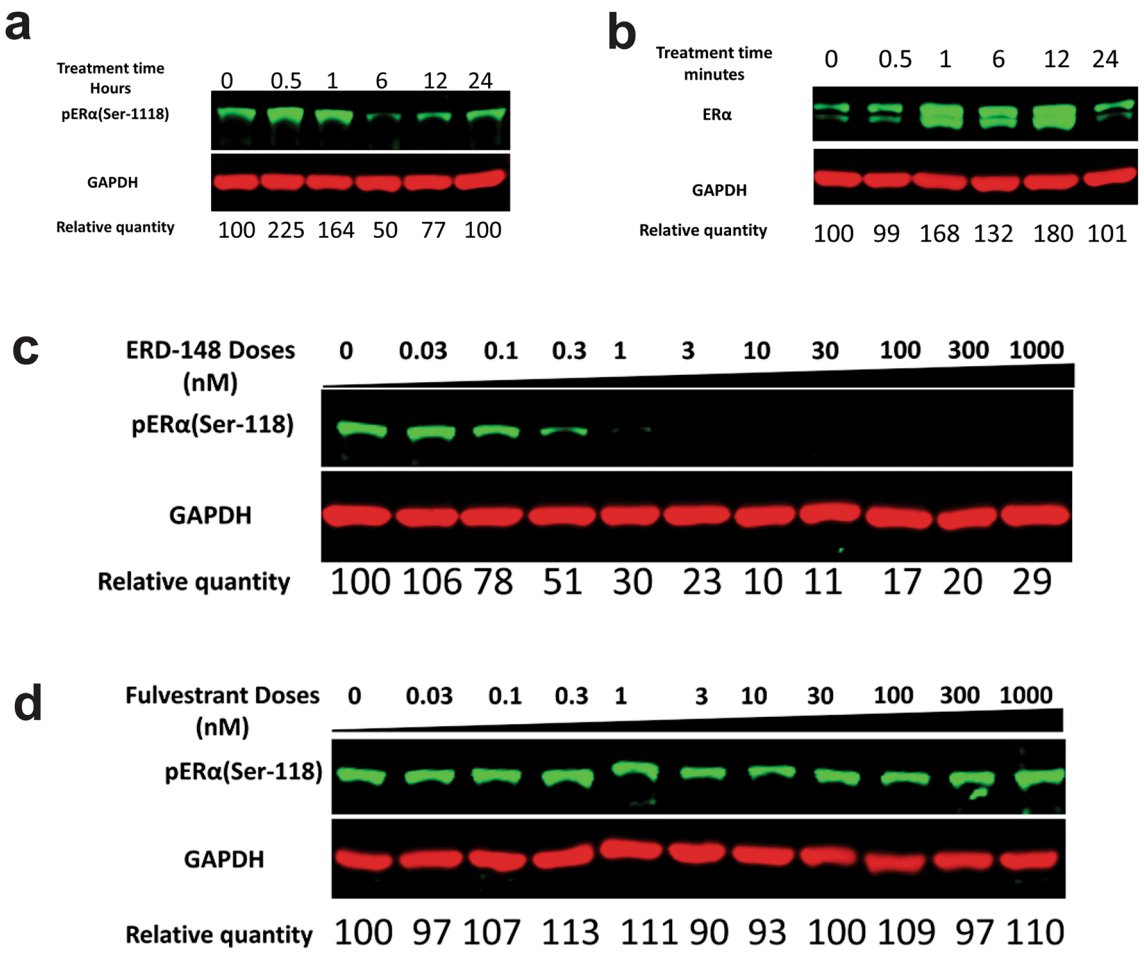

Fig. 2 Effect of ERD-148 and fulvestrant on Ser^{-118} phosphorylated ERα. **a** 17β-estradiol treatment increases both total ERα and Ser^{-118} phosphorylated ERα. MCF7 cells were treated with 100 pM 17β-estradiol at the indicated time. The level of Ser^{-118} phosphorylated ERα in the cell was evaluated by western blot analysis. **b** The total ERα in 17β-estradiol-treated samples was assessed by western blot analysis. **c** The MCF7 cells were treated with ERD-148 at indicated doses for 4 h. The effect of ERD-148 on Ser^{-118} phosphorylated ERα protein degradation is evaluated by western blotting analysis. **d** The MCF7 cells were treated with fulvestrant at indicated doses for 4 h. The effect of fulvestrant on Ser^{-118} phosphorylated ERα protein degradation is evaluated by western blotting analysis

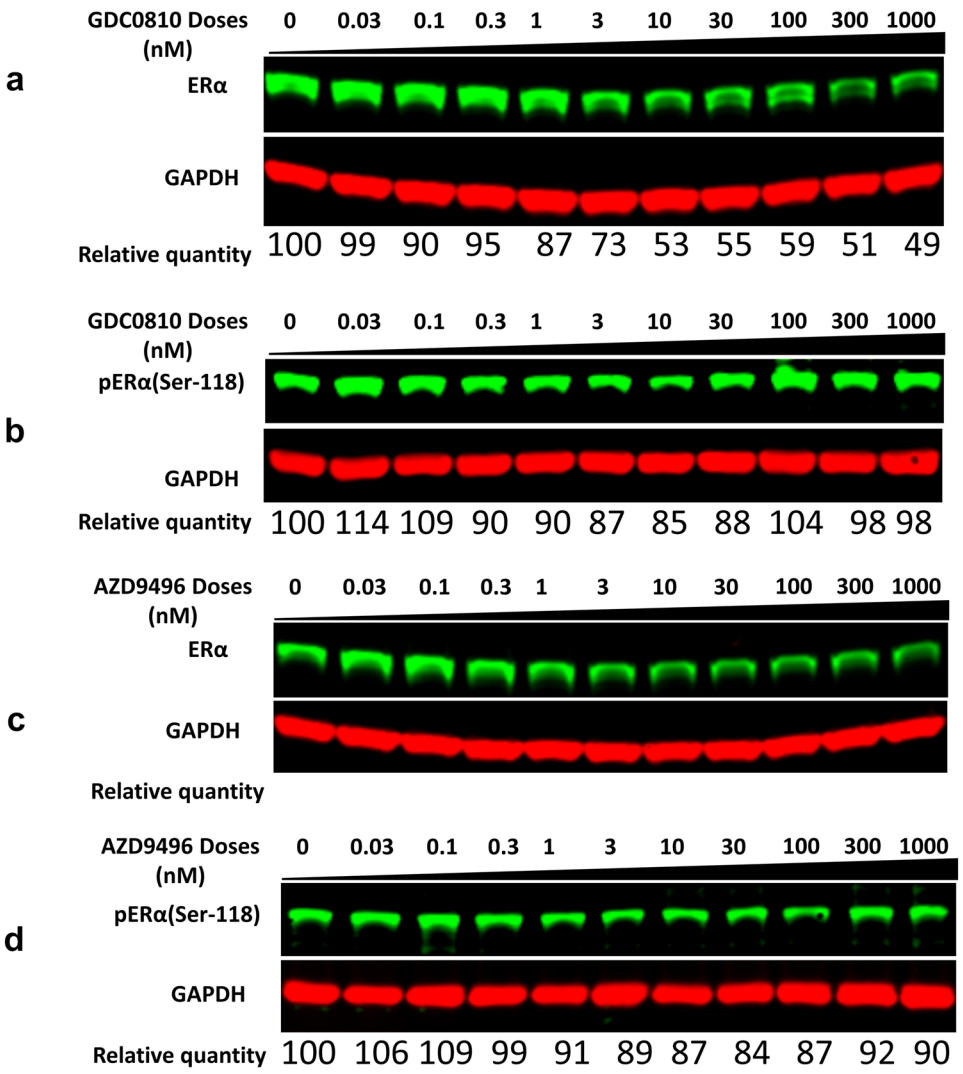

Fig. 3 Effect of GDC0810 and AZD9496 on the degradation of total ERα and ser^{-118} phosphorylated ERα. MCF7 cells were treated with either GDC0810 or AZD9496 at indicated doses for 4 h. The effect on total ERα and ser-^{118} phosphorylated ERα protein degradation is evaluated by western blotting analysis. **a** and **b** effect of GDC0810 on total ERα and ser^{-118} phosphorylated ERα degradation. **c** and **d** effect of AZD9496 on total ERα and ser^{-118} phosphorylated ERα degradation

by ERD-148 treatment (Fig. 4c). This result confirmed that PROTAC degraders degrade the ERα through different mechanisms as of fulvestrant.

Both compound ERD-148 and fulvestrant are ERα selective degraders

The MCF7 cells were treated with either fulvestrant or ERD-148 at doses ranging from 0 to 1 μM for 4 h as before. The samples were collected and analyzed by western blotting for ERβ gene expression. The result indicates that both compound ERD-148 and fulvestrant have a negligible effect on the ERβ degradation (Fig. 5), which confirms that both ERD-148 and fulvestrant are ERα-specific degraders.

ERD-148 inhibits MCF7 cell growth by inducing cell cycle arrest

The MCF7 and T47D cells were treated with indicated doses of ERD-148, fulvestrant, AZD9496, GDC0810, and raloxifene, respectively, and the cell growth was assessed by WST-8 assays. The data showed that ERD-148 is highly potent and effective in suppressing cell growth. It achieves an IC50 value of 1.4 nM and a maximum inhibition (Imax) of 61.7% in MCF7 cells (Fig. 6a), and an IC50 value of 0.77 nM and a maximum inhibition (Imax) of 60.5% in T47D cells (Fig. 6b). Fulvestrant is also highly effective and potent in inhibiting cell growth. It achieves an Imax value of 50.94% and 48.8% in MCF7 and T47D cells

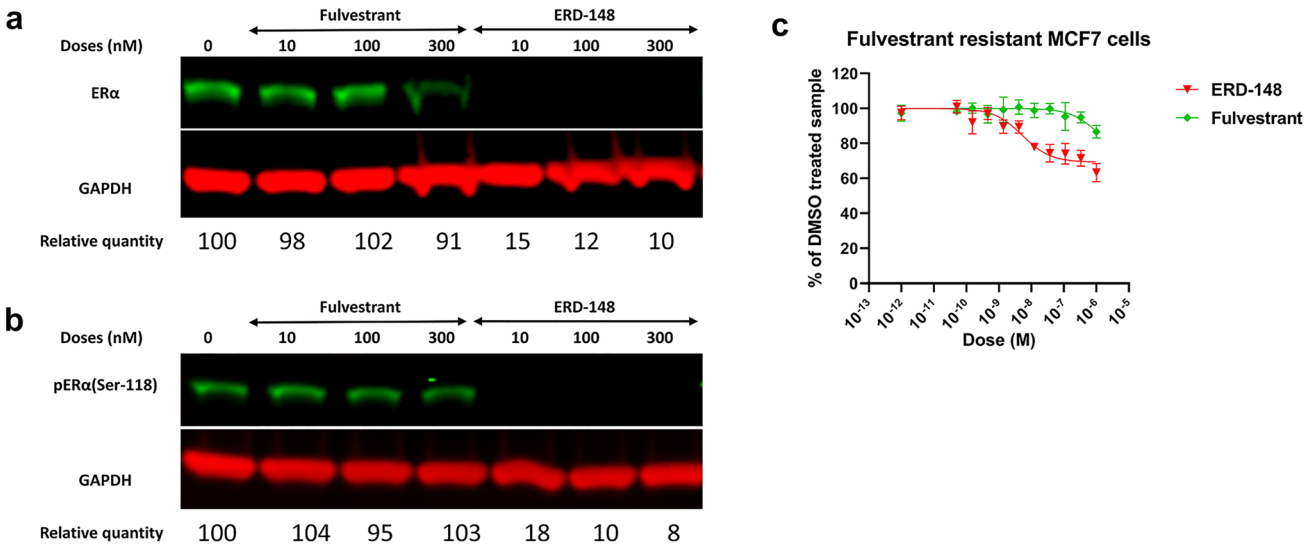

Fig. 4 ERD-148 degrades ERα in the fulvestrant-resistant MCF7 cells. Fulvestrant-resistant MCF7 cells were treated with fulvestrant or ERD-148 at indicated doses, respectively, for 4 h. The total ERα (**a**) and Ser-[118] phosphorylated ERα protein (**b**) levels were evaluated by western blotting analysis. (**c**). The cell growth of fulvestrant-resistant MCF7 cells was evaluated by WST-8 assays after compound treatment. Nonlinear regression fitting of the cell growth curves was conducted with GraphPad Prism software (version 9, by Graphstats Technologies). The data were presented as Mean ± SE from three independent experiments

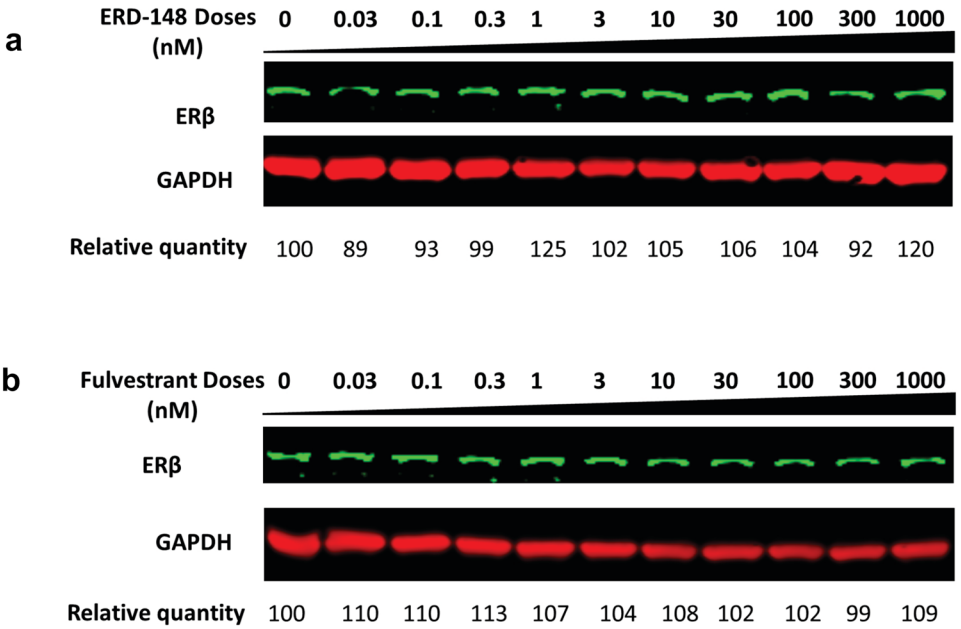

Fig. 5 Treatment of ERD-148 and fulvestrant on ERβ degradation. MCF7 cells were treated with either ERD-148 or fulvestrant at indicated doses for 4 h. The ERβ protein in total cell lysates is evaluated by western blotting analysis

(Fig. 6a and b). Raloxifene, on the other hand, achieves an Imax value of only 32.64% and 39.340%, respectively (Fig. 6a and b). Other SERDs, AZD9496 and GDC0810 achieve an Imax value of ~ 45% (Fig. 6a and b). Hence the more complete ER degradation achieved by ERD-148 translates into a greater maximum cell growth inhibition than that of fulvestrant, AZD9496, and GDC0810, three SERD molecules either approved or under clinical trials. Furthermore, both PROTAC degrader and SERDs achieve a much greater maximum cell growth inhibition than raloxifene, a SERM molecule.

The reduction of cell numbers after the compound treatment can be either due to cell death or the suppression of cell proliferation. Annexin V/PI staining is a widely used method to detect apoptotic cell death. Thus, we treated the MCF7 cells with 30 nM ERD-148, 30 nM fulvestrant, 1uM raloxifene, and the vehicle control, respectively, for 72 h. At these concentrations, the maximum degradation/inhibition

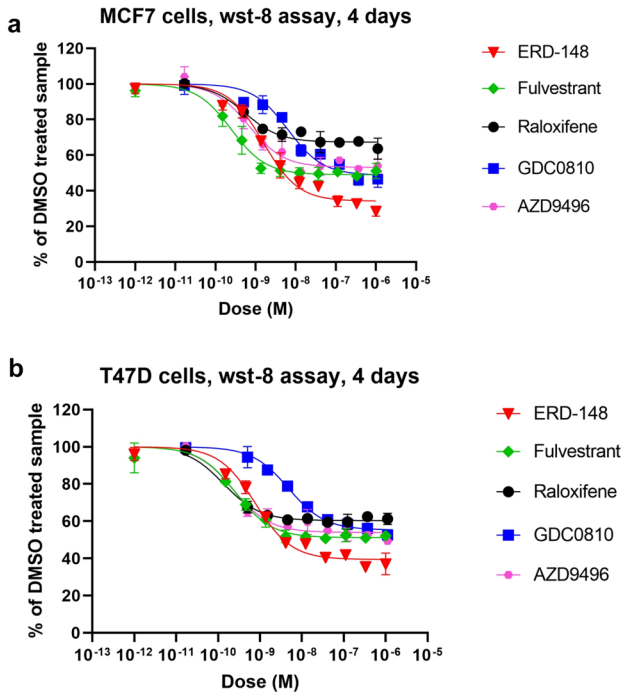

Fig. 6 Effect of compound treatment on MCF7 and T47D cell growth. MCF7 cells and T47D cells were treated with either vehicle or indicated compounds for four days. The cell growth was evaluated by WST-8 assay. Nonlinear regression fitting of the cell growth curves was conducted with GraphPad Prism software (version 9, by Graphstats Technologies). The data were presented as mean ± SE from three independent experiments

of ERα is achieved. The cells were stained with Annexin V/PI to evaluate the potential effect of these compounds on cell death by flow cytometry analysis. The results shown in Fig. 7 indicate that about 30% of the MCF7 cells were apoptotic cells after compound treatment (Fig. 7, Q 2 and Q3 combined). However, no significant increase was found for the dead cells in all the samples treated with compounds compared to the vehicle-treated controls (Fig. 7, Q 1 and Q2 combined).

We next treated the MCF7 cells for 48 h with the same set of compounds and evaluated if these treatments would induce cell cycle arrest. As shown in Fig. 8, the treatment of compounds decreased the cell population at the S stage. For ERD-148 and fulvestrant-treated samples, the cell population at the G1 stage increased dramatically as well after the treatment. The ERD-148 compounds-treated sample has the highest percentage of the G1 population (84% of the total cells) and lowest cell in the S stage (1.3% of the total cells), followed by fulvestrant-treated samples (72% of total cell in G1 and 9% in S stage) and by raloxifene-treated samples (64% of total cell in G1 and 11% of in S stage) (Fig. 8). The Increased percentage of the cells at the G1 stage and decreased percentage of the cells at the S stage are correlated with the maximum cell growth inhibition evaluated by the WST-8 assay. These results suggest that suppression of ERα in MCF7 cells mainly induces cell cycle arrest to inhibit the growth of MCF7 cells. PROTAC degrader ERD-148 is more efficient in suppressing the MCF7 tumor cell growth than the SERD fulvestrant and the SERM raloxifene.

ERD-148 has a similar gene regulation profile as fulvestrant

To further investigate the gene responses after the ERD-148 compound treatment, MCF7 cells were treated with 30 nM ERD-148, 30 nM fulvestrant, 1uM raloxifene, and the vehicle control. Eight hours after the treatment, the total RNAs were extracted and analyzed by gene microarray analysis.

Compound treatment leads to significant gene expression change. Upon ERD-148 treatment, 558 genes increased more than two-folds, and 602 genes decreased more than two-folds compared to their expression in vehicle-treated controls (Figs. 9a, b, and 10). The expression scatter plot between the ERD-148-treated sample and fulvestrant-treated samples showed that most genes fall inside the lines of twofold change (Fig. 9a). Very few genes (less than 0.5% of the tested genes) are outside the twofold line and showed significant differences in expression. Thus, although most of the gene expression levels varied a little bit between the ERD-148-treated samples and fulvestrant-treated samples, only a few genes showed significant variation in expression between ERD-148 and fulvestrant-treated samples. Both ERD-148 and fulvestrant suppress the ER signaling, cell cycle, and cell division pathways. In addition, a lot of genes in the PI3-AKT signaling pathway were elevated by these compounds. For SERM raloxifene-treated samples, more than 80% of the genes changed the same trend as in the SERD and PROTAC degrader-treated samples in terms of increase and decrease after compound treatment. ~200 genes are differently regulated compared to the degraders (Fig. 9b).

To confirm the result of gene microarray analysis, selected genes were assessed by qRT-PCR analysis. Consistent with the gene microarray result, *pGR, GREB1, AREG, EGR3,* and *JPH2* genes are strongly suppressed by ERD-148, fulvestrant, and raloxifene. *ANP32D* gene was significantly induced in the cells treated with these compounds (Fig. 11). The ERD-148 is only slightly more effective than

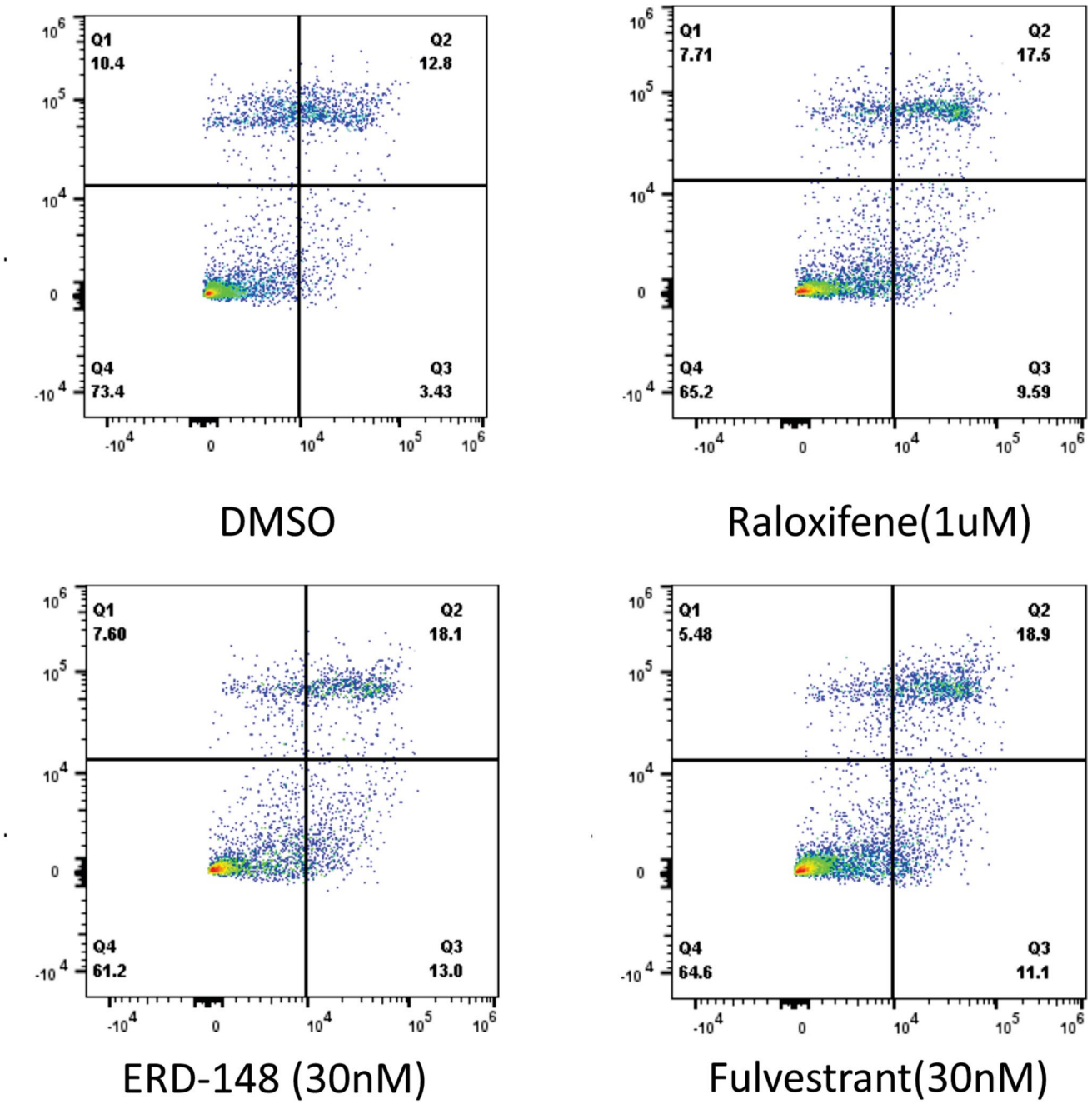

Fig. 7 Annexin V/PI staining of ERD-148-, fulvestrant-, and raloxifene-treated MCF7 cells. MCF7 cells were treated with either vehicle or indicated compounds, respectively, for 72 h. The cells were stained with AnnexinV/PI and separated by flow cytometry. Representative data from three independent experiments with the same results are shown in the figure

fulvestrant in suppressing the expression of *pGR* and *GREB1* at both 10 nM and 100 nM, but it is significantly more effective than raloxifene (Fig. 11). No significant change in *EGFR, ESR1*, and *ESR2* gene transcription was observed. *P53* is suppressed by the Raloxifene but not by the ERD-148 or fulvestrant (Fig. 11).

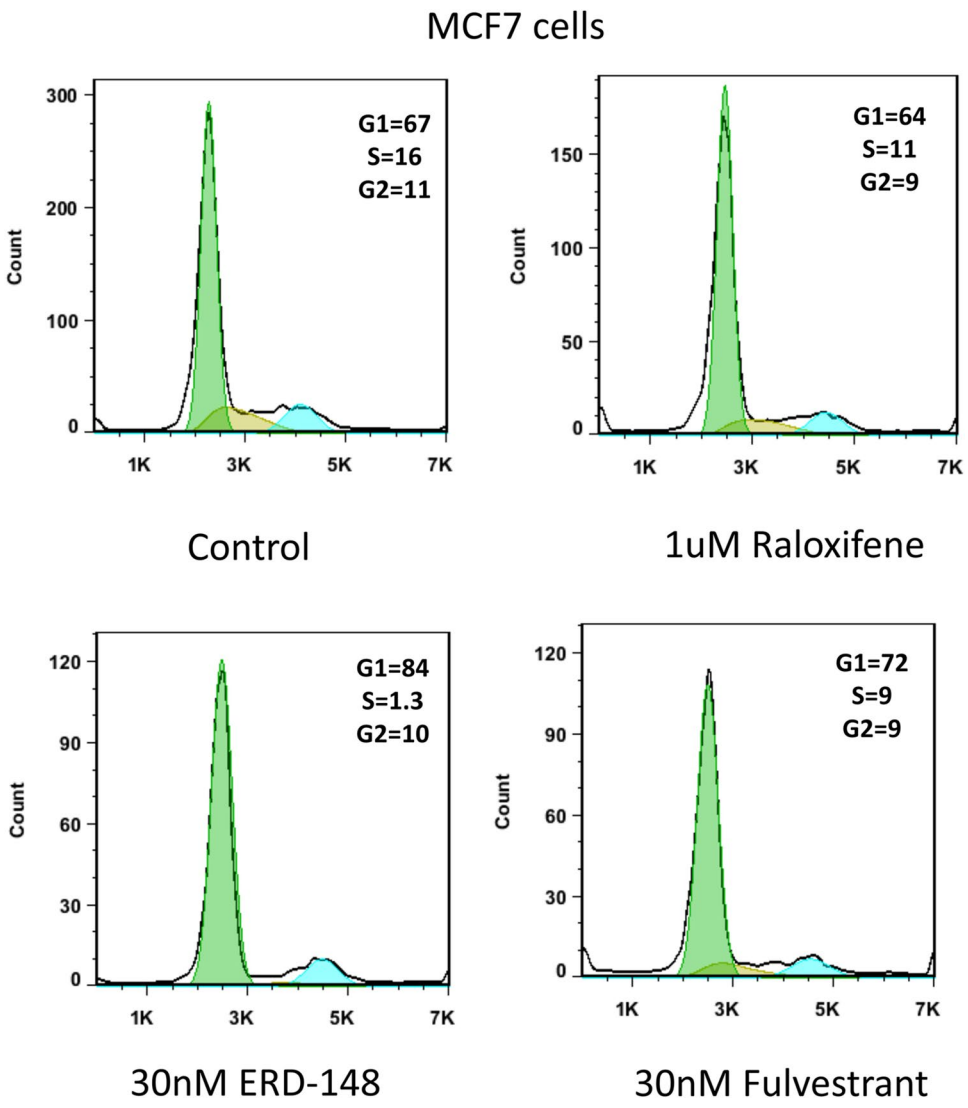

Fig. 8 Cell cycle analysis of ERD-148-, fulvestrant-, and raloxifene-treated MCF7 cells. MCF7 cells were treated with either vehicle or indicated compounds, respectively, for 48 h. The cells were fixed and stained with PI and analyzed by flow cytometry. Representative data from three independent experiments with the same results are shown in the figure

Discussion

Phosphorylation of ERα has been found on multiple amino acid residues[8]. The Ser^{-104}, Ser^{-106}, Ser^{-118}, and Ser^{-167} at the activation function 1 region of ERα N-terminal domain [8, 30] can be phosphorylated upon estradiol binding or in response to activation of the mitogen-activated protein kinase pathway[8]. The Ser^{-236} at the ERα DNA-binding domain is phosphorylated in response to the activation of protein kinase A[8]. The Tyr^{-537} at the ERα ligand-binding domain is phosphorylated by c-Src, which leads to the nuclear export of ERα [31]. Although many of these positions in the ERα protein can be phosphorylated, the human ERα is predominately phosphorylated at Ser^{-118} in response to estradiol binding [8]. In this study, we confirmed that the Ser^{-118} of ERα in cultured MCF7 is phosphorylated and is increased upon estradiol binding. However, we failed to detect any phosphorylation of ERα at other positions of ERα in MCF7 cells. It is interesting to observe that the degradation by PROTAC degrader ERD-148 is not affected by the phosphorylation of ERα, while all the SERD evaluated (including fulvestrant, AZD9496, and GDC0810) showed a dramatic decrease of degradation potency and maximum degradation to the phosphorylated form of ERα upon

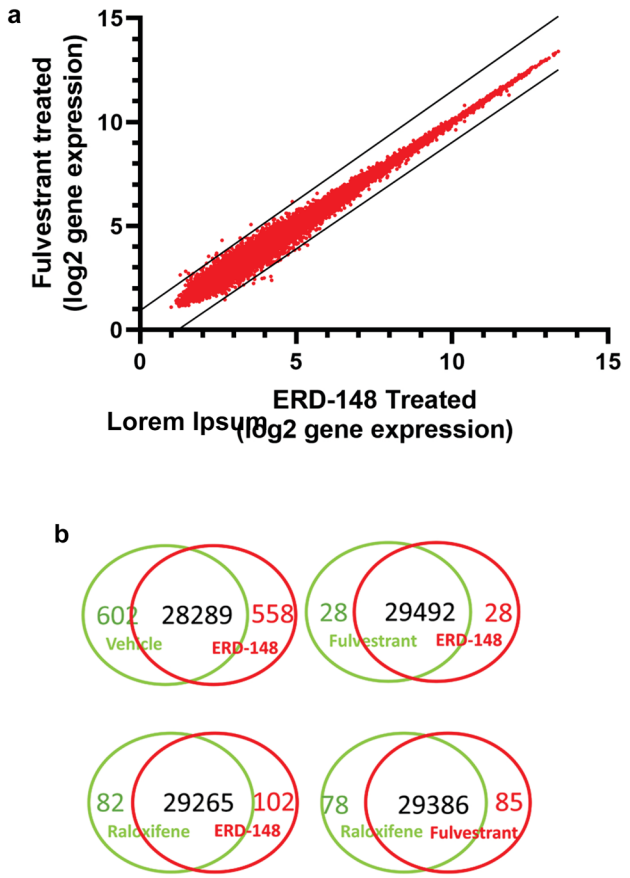

Fig. 9 Gene microarray analysis of ERD-148-, fulvestrant-, and Raloxifene-treated MCF7 cells. MCF7 cells were treated with either vehicle or indicated compounds, respectively, for 8 h. The total RNAs were extracted and analyzed by gene microarray analysis. **a** The expression value of 35,000 genes upon ERD-148 and fulvestrant treatment were compared against each other. The slanted lines indicated a twofold change of expression. **b** Comparison of gene expression between fulvestrant-, ERD-148-, and raloxifene-treated samples. Numbers with red, green, and black color indicate genes with more than two-fold increase, more than two-fold decrease, and less than two-fold change against the corresponding counterparts in the figure

treatment. One probable reason is that the phosphorylation of ERα affected the conformation of ERα. Unlike the SERD, which degrades the ERα through conformational change and induces misfolding of ERα, the activity of PROTAC ER degrader relies on the ligand-binding affinity of ERα. Thus, PROTAC ER degrader is less sensitive to the conformational change induced by ERα phosphorylation. Previous experiments with ERα mutant cell lines also confirmed that the potency of fulvestrant is more sensitive to protein conformation change[25].

Despite the PROTAC degrader and SERD degrader having different mechanisms to reduce the ERα in the cell, they all target the ERα protein to regulate the downstream gene expressions. Thus, the phenotypes of the cell after treatment are quite similar. More than 99% of genes followed the same trend between ERD-148- and fulvestrant-treated samples, although the extent of gene regulation is different, due to the difference in potency between these two types of compounds. This is reasonable as they both are highly selective ERα-specific degraders, only downstream ERα genes are affected upon the compound treatment.

Conclusions

Thus, our study indicated that ERD-148 degrades not only unphosphorylated ERα but also the phosphorylated ERα in the cells. The PROTAC ER degrader has a similar gene regulation profile as the SERD with different potency. The effect of growth inhibition by ERD-148 is mediated through the inhibition of cell proliferation. The finding that PROTAC ER degrader can degrade the ERα in fulvestrant-resistant cells provided evidence for the first time for the potential future clinical application in treating fulvestrant-resistant cancer.

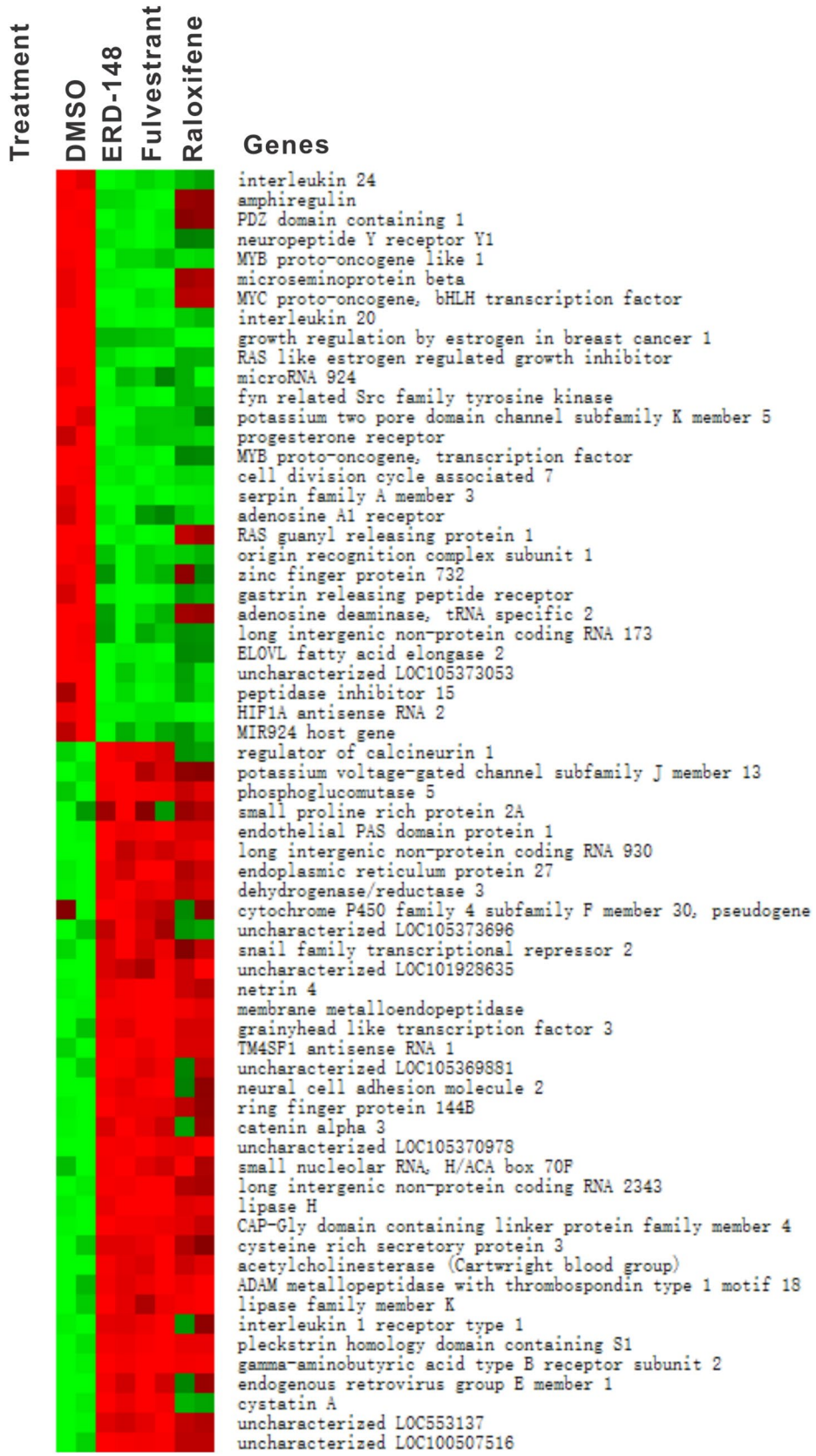

Fig. 10 Genes with more than fourfold changes against the vehicle-treated control were selected and shown in the heatmap. The red color indicates genes with increased expression and the green color indicates genes with decreased expression

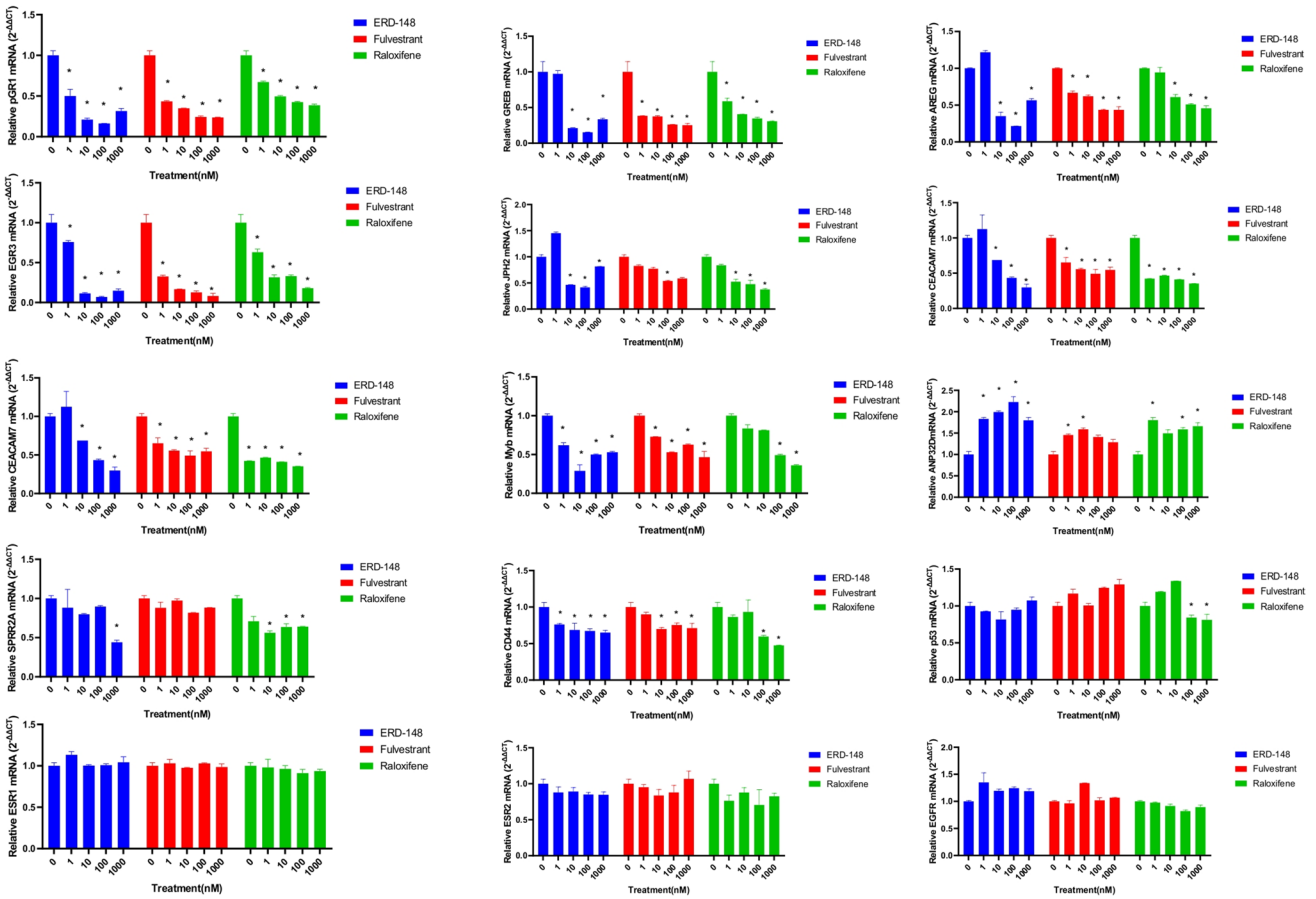

Fig. 11 Selected genes were assessed by real-time PCR analysis. The MCF7 cells were treated with indicated compounds for 8 h and the gene expression was tested by quantitatively RT-PCR analysis. A * indicates a significant difference ($P<0.05$) between the compound-treated sample and the vehicle-treated control sample. Data are shown as mean ± SE with $n=5$

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Data availability Data are available upon request.

Declarations

Competing interests The authors declare that they have no competing interests.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. The use of animals and cell lines was conducted following the NIH guidelines in the USA.

Consent for publication Not applicable.

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