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Diallyl trisulfide inhibits estrogen receptor-α activity in human breast cancer cells

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Abstract

Organosulfur compounds from garlic effectively inhibit growth of transplanted as well as spontaneous cancers in preclinical animal models without any adverse side effects. However, the mechanisms underlying anticancer effect of this class of compounds are not fully understood. This study reports, for the first time, that garlic organosulfide diallyl trisulfide (DATS) inhibits estrogen receptor-α (ER-β) activity in human breast cancer cells. Exposure of MCF-7 and T47D cells to DATS resulted in downregulation of ER-α protein, which peaked between 12- and 24-h post-treatment. DATS was relatively more effective in suppressing ER-α protein expression compared with its mono and disulfide analogs. The 17β-estradiol (E2)-induced expression of pS2 and cyclin D1, ER-α target gene products, was also decreased in the presence of DATS. Downregulation of ER-α protein expression resulting from DATS treatment was accompanied by a decrease in nuclear levels of ER-α protein, ER-α mRNA suppression, and inhibition of ERE2e1bluciferase reporter activity. DATS-mediated inhibition of cell viability and apoptosis induction were not affected in the presence of E2. In agreement with these results, ectopic expression of ER-α in MDA-MB-231 cell line failed to confer any protection against cell proliferation inhibition or apoptosis induction resulting from DATS exposure. DATS treatment caused a decrease in protein levels of peptidylprolyl *cis–trans* isomerase (Pin1), and overexpression of Pin1 partially attenuated ER-α downregulation by DATS. DATS-induced apoptosis was modestly but significantly augmented by overexpression of Pin1. In conclusion, this study identifies ER-α as a novel target of DATS in mammary cancer cells.

Keywords

Diallyl trisulfide; Breast cancer; Estrogen receptor-α; Chemoprevention

Introduction

Recorded history attests to the presence of health-promoting phytochemicals in garlic [1, 2]. Garlic and its metabolic byproducts are known to be beneficial against a number of chronic conditions, including cardiovascular disease, diabetes, infections, and cancer [1–5]. Anticancer effects of garlic and other *Allium* vegetables are ascribed to sulfur-containing compounds, which are either water- or lipid-soluble and generated after crushing or chewing of these vegetables [6]. Initial evidence for cancer protective role of *Allium* vegetables emerged from population-based case–control studies [7–10]. For example, meta-analysis of the association between raw and/or cooked garlic intake and the risk of colorectal cancer

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Conflict of interest None of the authors has any conflict of interest.

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revealed a relative risk estimate of 0.69 [10]. Similarly, after accounting for total caloric intake and other established risk factors, intake of garlic and onions was inversely associated with the risk of breast cancer in a French case–control study [9]. However, this association was not evident for garlic supplement use [8].

Previous studies have indicated that the lipid-soluble organosulfur compounds, particularly diallyl trisulfide (DATS), exhibit greater anticancer effect compared with water-soluble sulfur compounds (e.g., *S*-allylcysteine) [5, 11, 12]. Studies have also pointed toward a critical role for the allyl group and as well as the number of sulfur atoms in anticancer effect of oil-soluble organosulfur compounds [13, 14]. For example, studies from our own laboratory have revealed that DATS is relatively more potent in causing apoptotic cell death of human prostate cancer cells in comparison with diallyl sulfide (DAS) or diallyl disulfide (DADS) [13]. A similar structure–activity relationship was observed in MCF-7 human breast cancer cells [14].

DATS has been studied rather extensively for its in vivo anticancer efficacy in animal models [11, 12]. For example, oral treatment of female A/J mice with 20 μmol DATS prior to challenge with an environmental carcinogen (benzo[a]pyrene) resulted in about 85 % decrease in fore-stomach tumor multiplicity [15]. Oral administration of 6 μmol DATS (three times per week) to PC-3 human prostate cancer xenograft-bearing male athymic mice resulted in retardation of the tumor growth [16]. Growth retardation of MCF-7 human breast cancer xenografts in female Balb/c nude mice after 1-month oral treatment with 5 μmol DATS/kg (twice/week) was also documented [14]. The incidence of poorly differentiated prostate cancer was decreased significantly after oral administration of 2 mg DATS/mouse (three times/week orally) in a transgenic mouse model [17].

Recent studies have demonstrated anticancer effects of DATS against breast cancer [14, 18-20]. Studies from our laboratory indicated that DATS treatment resulted in apoptotic cell death in breast cancer cells (MCF-7, MDA-MB-231, and BRI-JM04) regardless of the p53 or human epidermal growth factor receptor-2 status [20]. We have also shown previously that a spontaneously immortalized and non-tumorigenic normal human mammary epithelial cell line (MCF-10A) is resistant to cell viability inhibition by DATS at 20 and 40 μM concentrations (24 h treatment) [20]. Unlike breast cancer cells, the MCF-10A cell line is also resistant to proapoptotic effect of DATS due to defects in Bak and Bax activation [20]. The proapoptotic effect of DATS in breast cancer cells was associated with reactive oxygen species-mediated activation of multidomain proapoptotic proteins Bax and Bak [20]. Another study implicated reactive oxygen species-dependent activation of c-Jun N-terminal kinase in apoptosis regulation by DATS in MCF-7 cells [14]. Furthermore, DATS was shown to abrogate benzo[a]pyrene-induced changes in MCF-10A cells [19]. This study shows, for the first time, that estrogen receptor-α (ER-α) is a novel target of DATS in mammary cancer cells.

Materials and methods

Reagents and cell lines

DADS (purity 98 %) and DATS (purity 96 %) were purchased from LKT Laboratories (St. Paul, MN). Other reagents, including DAS, 17β-Estradiol (E2), 4′,6-diamidino-2-phenylindole (DAPI), and propidium iodide were from Sigma-Aldrich (St. Louis, MO). Antibodies against ER-α, pS2, and cyclin D1 were from Santa Cruz Biotechnology (Dallas, TX); an antibody against peptidyl-prolyl *cis–trans* isomerase (Pin1) was from Cell Signaling Technology (Danvers, MA); and an antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from GeneTex (Irvine, CA). FuGENE6 transfection reagent and dual luciferase reporter assay kit were from Promega (Madison, WI). Reagents for

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reverse transcription-polymerase chain reaction (RT-PCR) were from Invitrogen-Life Technologies (Grand Island, NY). The MCF-7 and T47D human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in phenol red-free minimum essential medium (MCF-7) or RPMI 1640 (T47D and MDA-MB-231) containing 5 % charcoal/dextran-stripped fetal bovine serum (cFBS) to remove endogenous steroids for 2 days prior to the experiments. The ER-α overexpressing MDA-MB-231 cells have been previously described [21]. The Pin1-over-expressing MCF-7 cells were generated and maintained as previously described [22]. Cell culture media were purchased from Mediatech (Manassas, VA), whereas fetal bovine serum and antibiotic mixture were purchased from Invitrogen-Life Technologies. The cFBS was from HyClone-Thermo Fisher Scientific (Waltham, MA).

Western blotting

After treatment with dimethyl sulfoxide (DMSO), DAS, DADS, or DATS, cells were collected, and lysed as described previously [23]. Lysates were subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis for resolution of proteins followed by wet-transfer onto polyvinylidene fluoride membranes. The membranes were blocked with 5 % milk in tris-buffered saline containing 0.05 % Tween 20 and then incubated with the desired primary antibody. Membrane was washed with tris-buffered saline-containing 0.05 % Tween 20 and then treated with an appropriate secondary antibody. Immunoreactive bands were detected with the use of enhanced chemiluminescence reagent.

Determination of cell viability and cell proliferation

For viability assay, cells were seeded at a density of 1×10^5 cells per well in 12-well plates in triplicate and allowed to attach overnight. After desired treatment, cells were trypsinized and stained with trypan blue solution. Live cells were counted with the use of a hemocytometer. Cell proliferation was performed using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit from Promega (Madison, WI) according to the manufacturer's instructions.

Microscopic analysis of ER-α expression

MCF-7 and T47D cells were plated on coverslips, allowed to attach, and then treated with DMSO or 20 μM DATS for 6 or 12 h. Cells were then fixed with 2 % paraformaldehyde for 1 h at room temperature and permeabilized using 0.5 % Triton X-100 for 10 min followed by incubation with phosphate-buffered saline (PBS)-containing 0.5 % bovine serum albumin and 0.15 % glycine for 1 h. Subsequently, cells were treated with an anti-ER-α antibody at 4 °C for overnight followed by treatment with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes-Life Technologies) for 1 h at room temperature. After washing with PBS, cells were counterstained with DAPI for 5 min at room temperature to visualize nuclear DNA. Expression of ER-α was visualized with the use of a Leica DC300F fluorescence microscope at 100× objective magnification.

Luciferase reporter assay

MCF-7 and T47D cells were co-transfected with renilla and ERE2e1b-luciferase reporters (generous gift from Dr. Brian G. Rowan, Tulane University School of Medicine, New Orleans, LA) [24] prior to treatment with E2 (10 nM) and/or DATS (10 or 20 μM). Luciferase activity was determined using a luminometer as described previously [21].

RT-PCR for *ER-α* mRNA expression

RT-PCR was done to assess the effect of DATS treatment on *ER-α* mRNA level essentially as described previously [21]. In brief, after treatment of MCF-7 or T47D cells with DMSO

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(control) or varying doses of DATS, total RNA was extracted for synthesis of complementary DNA. The PCR products were resolved by 1.5 % agarose gel electrophoresis pre-stained with ethidium bromide. The primers and amplification conditions have been described before [21].

Determination of apoptosis

Apoptosis was determined by flow cytometric analysis of sub-diploid fraction (sub-G_0–G_1) as described by us previously [13] or by Annexin V-FITC/propidium iodide method using a kit from BD Biosciences. Desired cells ($2.5 \sim 3 \times 10^5$ cells per well) were seeded in six-well plates in triplicate, allowed to attach to the plates, and treated with DMSO or DATS for 24 h. Cells were then harvested by trypsinization and washed with PBS. Cells were resuspended in 100 μL of binding buffer, stained with 4 μL of Annexin V/FITC and 2 μL of propidium iodide solution for 15 min at room temperature in dark, and analyzed using a flow cytometer.

Results

DATS treatment downregulated ER-α protein expression in mammary cancer cells

Initially, we used MCF-7 cells and naturally occurring oil-soluble organosulfur compounds with varying sulfur atoms (Fig. 1a) to study the effect on ER-α protein expression by western blotting. DAS and DADS treatments had marginal impact on ER-α protein expression (Fig. 1b). On the other hand, DATS treatment resulted in a concentration-dependent decline in protein levels of ER-α (Fig. 1b). The inhibitory effect of DATS treatment on ER-α activity was confirmed by western blotting for its target gene products pS2 and cyclin D1. The E2-induced expression of ER-α target genes in MCF-7 and T47D cells was decreased markedly after 24 h treatment with 20 μM DATS (Fig. 1c). Time-course kinetic studies revealed dose-dependent downregulation of ER-α protein expression in the presence of DATS, which peaked between 12 and 24 h after treatment in MCF-7 as well as in T47D cells (Fig. 1d). These results indicated downregulation of ER-α protein upon treatment with DATS in human breast cancer cells.

We considered the possibility that DATS treatment may indirectly affect ER-α protein level due to cellular toxicity, but this study as well as published work argues against this likelihood. First, the expression of the loading control (GAPDH) was not decreased in MCF-7 or T47D (Fig. 1b, d). Second, our own work has revealed that the expression of heme oxygenase-1 protein is increased, not decreased, after 24 h treatment of MCF-7 cells with 20 and 40 μM DATS [20]. DATS treatment (50 μM, 12 h) has no meaningful impact on protein levels of Bad or p38 mitogen-activated protein kinase in MCF-7 cells [14]. There was no effect of DATS treatment even at 100 μM concentration (16 h treatment) on c-Jun N-terminal kinase and Bim protein levels in MDA-MB-231 breast cancer cells [25]. Nevertheless, we determined the effect of DATS treatment on MCF-7 and T47D cell viability after 6 and 12 h treatment. As can be seen in Fig. 1e, viability of these cells was minimally affected by DATS treatment at 6 h time point. However, the DATS-mediated decrease in ER-α protein level was evident after 6 h of treatment (Fig. 1d). Collectively, these findings indicated that downregulation of ER-α protein after DATS treatment was not a consequence of cellular toxicity.

DATS treatment decreased nuclear levels of ER-α protein in breast cancer cells

DATS-mediated decrease in ER-α protein was confirmed by microscopy. In DMSO-treated control MCF-7 (Fig. 2a) and T47D cells (Fig. 2b), the ER-α protein was found in both cytoplasm and nucleus but most of the ER-α protein was localized in the nucleus consistent with its role as a transcription factor. Downregulation as well as cytoplasmic re-distribution

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of ER-α protein was discernible after DATS treatment in both cell lines especially at the 12 h time point (Fig. 2a, b). Together these results indicated that suppression of ER-α protein expression after treatment with DATS in human breast cancer cells was not a cell line-specific phenomenon.

DATS treatment decreased transcription of ER-α

As can be seen in Fig. 3a, ERE2e1b-associated luciferase activity was increased significantly in the presence of E2 compared with DMSO-treated control. The E2-stimulated ERE2e1b-luciferase activity was inhibited significantly upon DATS treatment in MCF-7 and T47D cells (Fig. 3a). Furthermore, DATS treatment caused a decrease in mRNA levels of *ER-α* which peaked between 12 and 24 h similar to the protein expression changes (Fig. 3b). These results indicated suppression of *ER-α* transcription in DATS-treated breast cancer cells.

Effect of E2 on cell viability inhibition and apoptosis induction by DATS

DATS has been shown to inhibit viability of breast cancer cells including the MCF-7 cell line [14, 20]. We designed experiments to determine if cell viability inhibition by DATS was affected by E2 using MCF-7 cells. As expected, presence of E2 stimulated the expression of pS2, an E2-responsive gene product, in MCF-7 cells (Fig. 4a). As seen in Fig. 4b, 24 h treatment with DATS decreased the viability of MCF-7 and T47D cells. However, the cell viability inhibition by DATS treatment was not rescued in the presence of E2 (Fig. 4b). As shown in Fig. 4c, DATS treatment resulted in >2-fold enrichment of apoptosis in the absence of E2. Consistent with cell viability data, the DATS-induced apoptosis was not affected in the presence of E2 (Fig. 4c, d).

ER-α overexpression failed to confer protection against DATS-induced apoptosis in MDA-MB-231 cells

We next explored if DATS's antiproliferative and proapoptotic activities were affected by ER-α overexpression using MDA-MB-231cells, which lack expression of this receptor. Prior to the experiments, overexpression of ER-α was confirmed by western blotting (Fig. 5a). Similar to the results of the experiments using E2 (Fig. 4), ER-α over-expression did not have any meaningful impact on cell proliferation inhibition by DATS (Fig. 5b). Figure 5c depicts flow histograms for sub-G_0–G_1 fraction in MDA-MB-231 cells stably transfected with the empty vector or the same vector encoding for ER-α and treated for 24 h with DMSO or 20 μM DATS. In agreement with cell proliferation data (Fig. 5b), ER-α overexpression failed to confer any protection against DATS-mediated enrichment of sub-G_0–G_1 fraction (Fig. 5d). Similarly, the Annexin V/FITC method showed apoptosis induction by DATS treatment in both empty vector transfected cells and ER-α overexpressing MDA-MB-231 cells (Fig. 5e). These results indicated that, unlike certain other agents [21, 26], ER-α overexpression was dispensable for cell growth inhibition and apoptosis induction resulting from DATS exposure.

The role of Pin1 in ER-α downregulation by DATS

Recent studies have implicated Pin1 in regulation of ER-α stability and function [27–29]. Next, we raised the question of whether Pin1 level affected DATS-mediated suppression of ER-α protein expression. We addressed this question by overexpression of Pin1 in MCF-7 cells. The protein level of Pin1 was decreased after treatment with DATS (Fig. 6a). The downregulation of ER-α protein resulting from DATS treatment was markedly abrogated by Pin1 overexpression (Fig. 6a). However, the overexpression of Pin1 protein modestly but statistically significantly augmented DATS-induced apoptosis (Fig. 6b, c), which is not surprising because Pin1 is known to regulate function of other proteins [30].

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Discussion

Estrogens promote growth of breast cancer via ER-α signaling [31, 32]. The ER-α belongs to the nuclear receptor superfamily and primarily functions as a ligand-activated transcription factor-regulating expression of various genes [31, 32]. ER-α is expressed in about 70 % of human breast cancers [31]. Selective estrogen receptor modulators (e.g., tamoxifen and raloxifene) represent standard of care for ER-α positive breast cancers [33, 34]. This study shows, for the first time, that a non-toxic metabolite of processed garlic (DATS) suppresses ER-α expression and its function in human breast cancer cells. However, the downregulation of ER-α by DATS treatment seems transient as some reversibility of this effect is observed at the 24 h time point.

The results presented herein indicate that the cell growth inhibition by DATS is not rescued after estradiol stimulation (ER activation) in MCF-7 cells (Fig. 4b) or by ER-α overexpression in MDA-MB-231 cells (Fig. 5b), which should be viewed as a therapeutic advantage as ER-α is overexpressed in a large fraction of human breast cancers [31]. The results on DATS (this study) are in sharp contrast to another naturally occurring chemopreventive agent (withaferin A) with which the cell viability inhibition is significantly rescued after E2 stimulation in MCF-7 cells [21]. In another study, ER-α conferred resistance to paclitaxel in breast cancer cells through inhibition of apoptotic cell death [26]. DATS-induced apoptosis, on the other hand, is not affected by E2 or ER-α overexpression.

DATS concentrations necessary for suppression of ER-α expression and function may be pharmacologically achievable based on pharmacokinetics in rats after a single injection with 10 mg DATS [35]. Peak blood concentration of DATS was shown to be 5516.9 μg/L which equates to about 31 μM DATS [35]. It is important to point out that DATS has also been tested clinically for its safety profile and chemopreventive effect [36]. Oral administration of 200 mg of synthetic DATS (allitridum) every day in combination with 100-μg selenium every other day for 1 month of each of the 3 years of treatment was well-tolerated by all subjects [36]. In the first 5 year follow-up after stopping the treatment, a decrease in cancer morbidity was reported for the treatment group [36].

Global gene expression profiling after E2 treatment in MCF-7 cells showed robust changes in expression of over 400 genes, and a large fraction of these genes (about 70 %) were downregulated after E2 exposure [37]. The upregulated genes included survival promoters including survivin, multiple growth factors, and genes involved in cell cycle control [37]. Interestingly, E2-treated MCF-7 cells exhibited downregulation of several apoptosis-linked genes such as BCL-2 antagonist/killer 1 (Bak) and caspase 9 [37]. We have shown recently that apoptosis induction by DATS in breast cancer cells is mediated by activation of Bak protein [20]. The results of this study indicate that the DATS-induced apoptosis is not affected in the presence of E2. These results have clinical ramifications to suggest that DATS can overcome ER-α activation or overexpression in execution of apoptotic cell death.

Pin1 has been shown to positively regulate the expression of ER-α protein in breast cancer cells by inhibiting its proteasomal degradation [28]. Pin1 expression also correlates with ER-α protein level, but not its transcript level, in clinical specimens of human breast tumor [28]. Moreover, Pin1 overexpression is associated with tamoxifen resistance in MCF-7 cells due to epithelial–mesenchymal transition [38]. This study reveals that DATS treatment decreases protein level of Pin1 in MCF-7 cells, and Pin1 overexpression partially rescues DATS-mediated suppression of ER-α protein. Interestingly, we have also shown previously that DATS treatment inhibits epithelial–mesenchymal transition in breast cancer cells [20]. Thus, it is possible that Pin1 downregulation may contribute to DATS-mediated inhibition of

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epithelial–mesenchymal transition, but further work is necessary to experimentally test this possibility.

Clinically used selective ER modulators have serious side effects [33, 39]. Increased risk of uterine cancer, thromboembolism, cataracts, and perimenopausal symptoms are the well-documented adverse effects for selective ER modulators [33, 39]. Emergence of resistance is another limitation of these agents [38]. On the other hand, safety of DATS has been established in preclinical rodent models [14, 16, 17] as well as in a human interventional study [36]. Thus, DATS may represent a safer alternative to target ER-α for possible prevention of human breast cancer.

The salient findings of this study are: (a) a non-toxic compound from processed garlic (DATS) inhibits expression and activity of ER-α in breast cancer cells, and this effect is not a cell line-specific phenomenon; (b) the oligosulfide chain length is important for ER-α protein suppression by this class of compounds; (c) unlike certain other agents [21, 26], DATS can overcome ER-α activation and its overexpression for the execution of apoptosis in breast cancer cells; (d) Pin1 partially accounts for ER-α protein suppression resulting from DATS exposure at least in MCF-7 cells; and (e) Pin1 overexpression modestly augments DATS-induced apoptosis.

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Abbreviations

cFBS	Charcoal/dextran-stripped fetal bovine serum
DAS	Diallyl sulfide
DADS	Diallyl disulfide
DATS	Diallyl trisulfide
DAPI	4′,6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
E2	17β-Estradiol
ER-α	Estrogen receptor-α
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
PBS	Phosphate-buffered saline
Pin1	Peptidyl-prolyl *cis–trans* isomerase
RT-PCR	Reverse transcription-polymerase chain reaction

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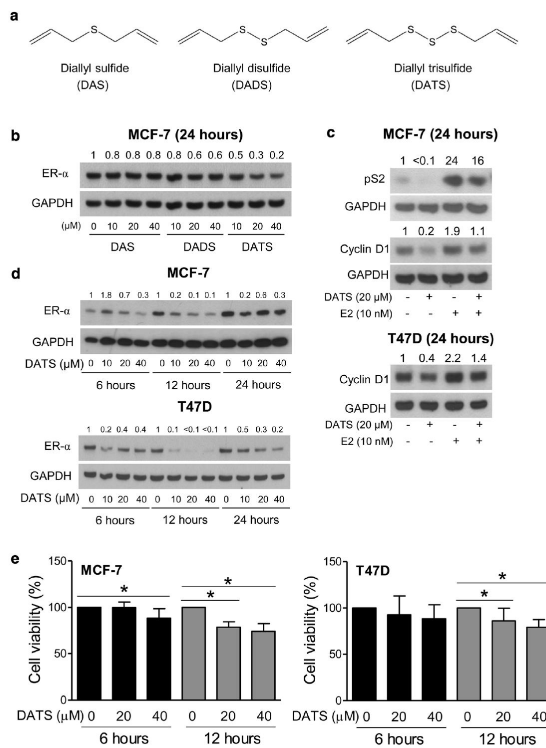

Fig. 1.

DATS treatment suppresses ER-α protein expression in MCF-7 and T47D cells. **a** Chemical structures of the organosulfur compounds used in this study. **b** Western blotting for ER-α protein using lysates from MCF-7 cells treated for 24 h with DMSO or the indicated doses of DAS, DADS, and DATS. **c** Western blotting for estrogen-inducible proteins in MCF-7 and T47D cells after 24 h treatment with DATS (20 μM) and/or E2 (10 nM). **d** Western blotting for time-course kinetic effect of DATS treatment on ER-α protein level in MCF-7 and T47D cells. Densitometric quantitation of band intensity relative to corresponding DMSO-treated control after normalization for loading control is shown on top of the band. e Effect of DATS treatment (6 and 12 h) on viability of MCF-7 and T47D cells. Combined results from two independent experiments are shown as mean ± SD (n = 6). *Significantly different between the indicated groups by one-way ANOVA followed by Dunnett's test. Each experiment was performed at least twice and the results were consistent

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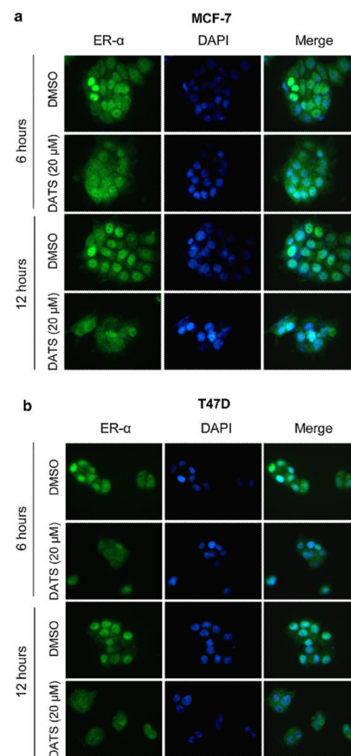

Fig. 2.

DATS treatment decreases nuclear levels of ER-α protein in MCF-7 and T47D cells. Immunocytochemical analysis of ER-α protein level in **a** MCF-7 cells and **b** T47D cells after 6 or 12 h treatment with DMSO or 20 μM DATS (×100 objective magnification). Similar results were observed in replicate experiments

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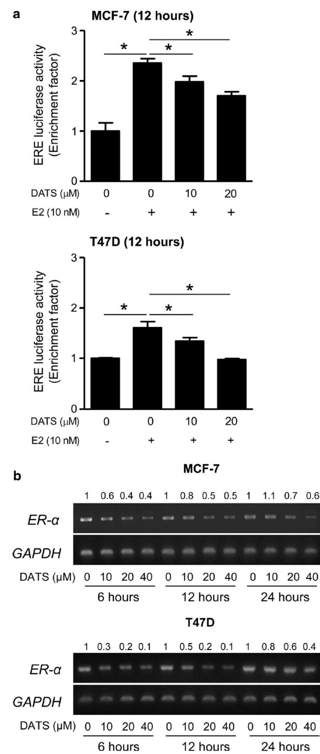

Fig. 3.

DATS treatment decreases *ER-α* mRNA expression in MCF-7 and T47D cells. **a** ERE2e1b-associated luciferase reporter activity in MCF-7 and T47D cells after 12 h treatment with 10 nM E2 alone or E2 plus DATS (10 or 20 μM). Results shown are mean ± SD ($n = 3$). *Significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's test. **b** RT-PCR for *ER-α* mRNA expression in MCF-7 and T47D cells after 6, 12, or 24 h treatment with DMSO or the indicated concentrations of DATS. Number above band indicates change in mRNA level relative to corresponding DMSO-treated control after normalization to *GAPDH* mRNA level. Each experiment was repeated at least twice

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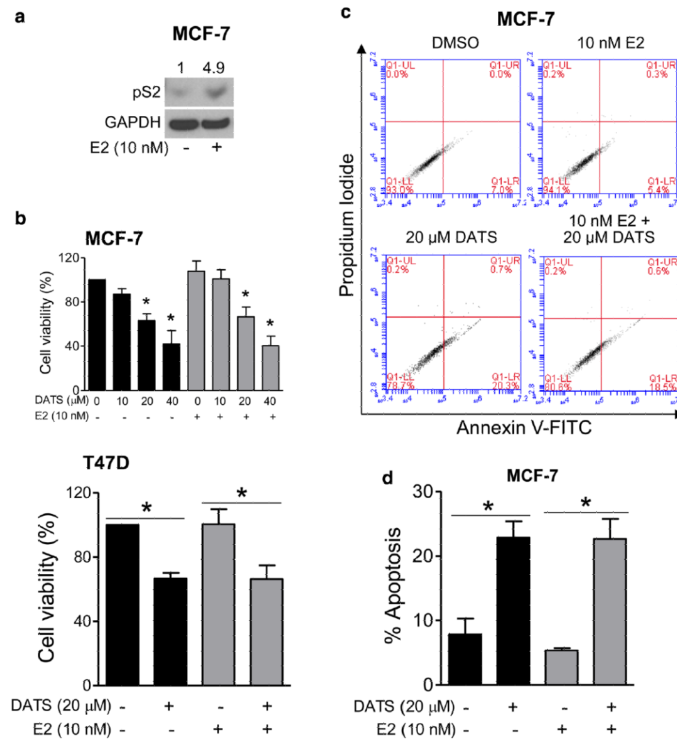

Fig. 4.

Effect of E2 on DATS-induced apoptosis. **a** Western blotting for estrogen-inducible pS2 protein in MCF-7 cells with or without treatment with 10 nM E2. **b** Cell viability was determined by trypan blue dye exclusion assay using MCF-7 and T47D cells treated for 24 h with DMSO or indicated doses of DATS in the absence or presence of E2. Results shown are mean ± SD ($n = 3–6$). *Significantly different ($P < 0.05$) compared with corresponding DMSO-treated control by one-way ANOVA followed by Bonferroni's test. **c** Representative flow histograms showing early (Annexin V positive) and late apoptotic fractions (Annexin V + propidium iodide positive) in MCF-7 cells treated for 24 h with DMSO or 20 μM of DATS in the absence or presence of 10 nM E2. **d** Quantitation of early + late apoptosis from data shown in **c**. Results shown are mean ± SD ($n = 3$). *Significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's test. Similar results were observed in replicate experiments

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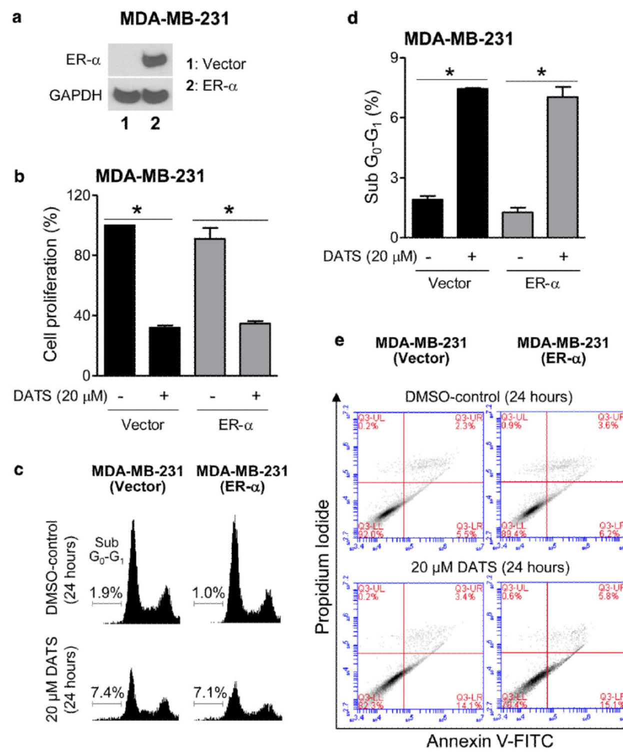

Fig. 5.

Overexpression of ER-α fails to confer protection against DATS-induced apoptosis in MDA-MB-231 cells. **a** Western blotting for ER-α protein using lysates from empty vector transfected cells (*lane 1*) and ER-α overexpressing MDA-MB-231 cells (*lane 2*). **b** Cell proliferation assay was done using empty vector-transfected control cells and ER-α overexpressing MDA-MB-231 cells after 72 h treatment with DMSO or 20 μM of DATS. Results shown are mean ± SD ($n = 3$). *Significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's test. **c** Representative flow histograms depicting sub-G_0–G_1 phase populations in empty vector transfected and ER-α overexpressing MDA-MB-231 cells treated for 24 h with DMSO (control) or 20 μM of DATS. **d** Quantitation of sub-G_0–G_1 phase population from data shown in **c**. *Significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's test. e Representative flow histograms showing early and late apoptosis in empty vector transfected and ER-α-overexpressing MDA-MB-231 cells treated for 24 h with DMSO or 20 μM of DATS. Each experiment was performed at least twice and the results were consistent

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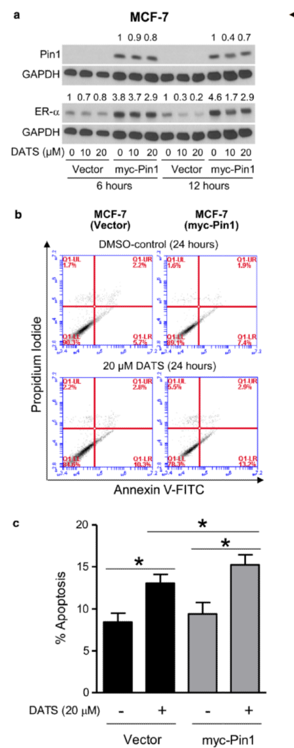

Fig. 6.

DATS-induced apoptosis is modestly augmented by Pin1. **a** Western blotting for Pin1 and ER-α proteins using lysates from empty vector transfected and Pin1-overexpressing MCF-7 cells treated with the indicated doses of DATS or DMSO for 6 and 12 h. Densitometric quantitation of band intensity relative to corresponding DMSO-treated control after normalization to loading control is shown on top of the band. **b** Representative flow histograms showing early and late apoptosis in empty vector transfected and Pin1-overexpressing MCF-7 cells treated for 24 h with DMSO or 20 μM of DATS. **c** Quantitation of apoptosis from results shown in panel **b**. Combined results from two independent experiments are shown as mean ± SD ($n = 6$). *Significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's test