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Phytoestrogen regulation of a Vitamin D_3 receptor promoter and 1,25-dihydroxyvitamin D_3 actions in human breast cancer cells☆

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Abstract

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), a steroid hormone derived from Vitamin D_3, is a negative growth regulator of breast cancer cells, and Vitamin D_3 analogs represent a novel treatment approach for human cancer. Elucidation of Vitamin D_3 receptor (VDR) regulation may reveal strategies to sensitize cancer cells to the effects of 1,25-dihydroxyvitamin D_3 and Vitamin D_3 analogs. We have previously characterized an estrogen responsive promoter region (800 bp upstream of exon 1c) in the human VDR gene, and the present studies examined regulation of this VDR promoter region by two phytoestrogens, resveratrol (present in red wine) and genistein (present in soy). We transiently transfected a VDR promoter luciferase construct into the estrogen receptor (ER) positive human breast cancer cell lines T47D and MCF-7, and treated with 0.4–4 μM resveratrol or 5–500 nM genistein. Both phytoestrogens up-regulated the transcription of the VDR promoter, as measured by reporter gene activity, approximately two-fold compared to vehicle treated cells. Co-treatment with the anti-estrogen tamoxifen (TAM) in T47D cells and transfection in an estrogen receptor negative breast cancer cell line demonstrated that the effects of phytoestrogens on the VDR promoter are dependent on estrogen receptor. Resveratrol and genistein also increased VDR protein expression as detected by Western blotting. Treatment with resveratrol had no effect on cell number or cell cycle profile, while treatment with genistein increased cell number. Because resveratrol could up-regulate VDR without increasing breast cancer cell growth, we hypothesized that resveratrol mediated increase in VDR expression would sensitize breast cancer cells to the effects of 1,25-dihydroxyvitamin D_3 and Vitamin D_3 analogs. In support of this hypothesis, both T47D and MCF-7 cells pre-treated with resveratrol exhibited increased VDR mediated transactivation of a Vitamin D_3 responsive promoter compared to cells pre-treated with vehicle. In addition, co-treatment with resveratrol enhanced the growth inhibitory effects of 1,25-dihydroxyvitamin D_3 and the Vitamin D_3 analog EB1089. These data support the concept that dietary factors, such as phytoestrogens, may impact on breast cancer cell sensitivity to Vitamin D_3 analogs through regulation of the VDR promoter.

Keywords: Vitamin D_3; Vitamin D_3 receptor (VDR); Resveratrol; Genistein; Phytoestrogens; Breast cancer

1. Introduction

The steroid hormone 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) binds the Vitamin D_3 receptor (VDR) and this ligand/receptor complex regulates the transcription of genes involved in cell cycle, apoptosis and differentiation. In breast cancer cells, $1,25(OH)_2D_3$ causes growth arrest and apoptosis in vitro and in vivo [1], suggesting that Vitamin D_3 based therapeutics may be useful for human cancer. Since the levels of $1,25(OH)_2D_3$ needed to achieve growth regulation can induce hypercalcemia, structural analogs such as EB1089 have been developed which display increased cell regulatory effects with minimal side effects [2]. EB1089 has been shown to decrease tumor growth in animal models and is now in clinical trials for human cancers [3]. Studies have shown that 80% of human breast tumors express VDR, but the expression of VDR may be at low levels [4]. Since the magnitude of a patient's response to Vitamin D_3 analog treatment could be limited by the level of VDR expression, strategies that enhance VDR expression might enhance the effectiveness of Vitamin D_3 based therapies.

In studies to define the molecular regulation of the VDR, our lab identified a novel promoter region upstream of exon 1c in the human VDR gene. This VDR promoter region drives expression of a luciferase reporter gene and is up-regulated by hormones and growth factors in breast cancer cells. Specifically, exon 1c VDR promoter activity

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is up-regulated in breast cancer cells by estrogen in an estrogen receptor (ER) dependent manner [5]. Sequence data revealed that although no consensus estrogen response elements (ERE) are present in this VDR promoter, several Sp1 transcription factor sites, which can confer estrogen responsiveness in other promoter contexts, were identified. In the studies reported here, we have used this hormone responsive VDR promoter to screen for estrogen-like compounds that could up-regulate VDR signaling in breast cancer cells. We studied the effects of two phytoestrogens, genistein (GEN) and resveratrol (RES) on VDR signaling in two ER positive human breast cancer cell lines, MCF-7 and T47D. These phytoestrogens have attracted considerable interest, particularly on the part of consumers, as potential alternatives to estrogen replacement therapy for relief of menopausal symptoms. Like natural estrogen, phytoestrogens interact with both ERα and ERβ subtypes (usually with lower affinity than that of estrogen) and many can activate estrogen responsive genes containing both classical EREs and Sp1 sites [6,7]. However, the molecular effects of phytoestrogens on breast cancer cells, particularly at low concentrations that are physiologically achievable, are not well defined. Although many phytoestrogens, including RES and GEN, are available without prescription as dietary supplements, there is limited experimental data on which to base recommendations regarding the use of phytoestrogens in post-menopausal women.

Studies have shown that the circulating concentration of GEN in rats and human subjects ranges from 5 nM to 1 μM [8,9]. At these concentrations, GEN mimics the proliferative effect of estrogen in T47D and MCF-7 cells (reviewed in [7]). RES (3,5,4′-trihydroxystilbene) is present in red wine at concentrations ranging from 0.05 to 14.5 mg/l [6] and circulating levels of approximately 4 nM RES have been detected in rats fed RES at doses equivalent to the amount present in two glasses of red wine [10]. While it has been reported that RES inhibits growth of breast cancer cells in vitro [11,12] and prevents pre-neoplastic lesions in mammary gland organ culture [13], these effects are observed at μM doses of RES, which are much higher than those which can be achieved in vivo after dietary ingestion. Furthermore, at some doses, RES mimics the proliferative effects of estrogen on breast cancer cells [14].

In the present studies with breast cancer cells, we demonstrate that both GEN and RES up-regulate the exon 1c VDR promoter and enhance steady state VDR protein expression in an ER-dependent manner. Phytoestrogen regulation of the VDR promoter was similar in T47D and MCF-7 cell lines, which express both ERα and ERβ subtypes [15]. In both cell lines, RES up-regulates VDR expression at nM concentrations (which do not stimulate proliferation), enhances VDR mediated transcriptional activation and sensitizes cells to the growth inhibitory effects of $1,25(OH)_2D_3$. These data support the novel concept that phytochemicals can enhance breast cancer cell sensitivity to Vitamin D_3 based therapeutics via up regulation of the VDR.

2. Materials and methods

2.1. Cell culture and growth assays

T47D and MCF-7 human breast cancer cells (obtained from ATCC) were routinely cultured in RPMI media supplemented with 5% fetal bovine serum. For all experiments described here, cells were plated in RPMI media containing 1% FBS. For assessment of relative cell number by crystal violet assay, cells plated in 12-well dishes were treated with phytoestrogens, 17β-estradiol, 4-hydroxytamoxifen (TAM), $1,25(OH)_2D_3$ and/or the synthetic Vitamin D_3 analog EB1089 (Leo Pharmaceuticals, Ballerup, Denmark) at concentrations indicated in the figure legends. Adherent cell numbers were assessed in plates fixed with 1% gluteraldehyde, stained with 0.1% crystal violet, rinsed and dried. Stain was solubilized in 1% Triton X100 and absorbance, which is proportional to total adherent cell number, was read at 590 nm on the Wallac Victor 2 plate reader.

2.2. Transient transfections

Cells (2×10^5 per well) were plated in 6-well dishes and incubated overnight. FuGENE 6 transfection reagent (Roche) was used following manufacturer's instructions to transfect cells with either empty vectors (Promega, Madison, WI) or vectors containing the 5′ flanking region of exon 1c of the human VDR promoter (pRL-800), a 300 bp region of the Vitamin D_3 responsive 24-hydroxylase promoter [pGL-24(OH)] or the estrogen responsive p3-ERE-TAT-luciferase reporter (pGL-ERE). The pRL-800 human VDR promoter vector was constructed in our lab [5], the pGL-24(OH) vector was provided by Dr. J. Omdahl (University of New Mexico), and the pGL-ERE vector was provided by Drs. R. Hilf and R. Bambara (University of Rochester) and Dr. C. Klinge (University of Louisville School of Medicine). Vectors containing an SV40-driven luciferase gene (Promega) were co-transfected with experimental vectors to normalize for transfection rate. Cells were incubated overnight in media containing transfection mix and treatments, harvested, and assayed with the Dual Luciferase kit (Promega).

2.3. Western blot analysis

Cells treated for 48 h with RES or GEN were lysed in KTED (300 mM sodium chloride, 10 mM Tris, 1 mM EDTA, 10 mM sodium molybdate, pH 7.5) buffer containing protease and phosphatase inhibitors (10 mM benzamidine, 10 mM sodium fluoride, 100 mM sodium vanadate, 25 μg/μl leupeptin, 25 μg/μl aprotinin). Proteins were precipitated from high salt nuclear extracts and total protein was analyzed using the BioRad coomassie blue dye reagent. Equal amounts of total protein were separated on 10% SDS–PAGE gels and transferred to nitrocellulose. The membranes were blotted with monoclonal anti-VDR (9A7, Neomarkers)

followed by horseradish peroxidase-conjugated anti-rat IgG (Amersham) and developed with the chemiluminescence.

2.4. Flow cytometry

For cell cycle analysis, cell monolayers were washed with PBS, trypsinized, pelleted and fixed in 90% ethanol at −20 °C. Fixed cells were incubated with propidium iodide (Sigma)/RNAse (Roche Molecular Biochemicals) solution for 20 min, and DNA histograms were obtained on 10^4 cells in a Beckman Coulter Epics XL flow cytometer. DNA histograms were modeled with the Multiplus AV software (Phoenix Flow Systems).

2.5. Data analysis

Statistical evaluation was by one-way analysis of variance (ANOVA), followed by multiple comparison tests (Dunnett or Tukey as appropriate) with Graph Pad Prism software version 3.00 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com. Differences between means were considered significant if *P*-values less than 0.05 were obtained.

3. Results

3.1. Effect of phytoestrogens on VDR promoter activity and VDR expression in breast cancer cells

The first goal of these studies was to assess whether RES or GEN could mimic the ability of estrogen to up-regulate the exon 1c VDR promoter [5]. This possibility was tested by transient transfection assays with a luciferase reporter vector that contains the 800 bp region immediately upstream of exon 1c in the VDR gene (pRL-800). The activity of the pRL-800 luciferase reporter gene was significantly higher than that of the pRL-null (empty) vector in both T47D cells (Fig. 1A) and MCF-7 cells (Fig. 1B), indicating basal activity of this VDR promoter in these two breast cancer cell lines. To test whether phytoestrogens altered VDR promoter activity, cells were treated for 18 h with RES at concentrations similar to those achievable in vivo after dietary ingestion. In both T47D and MCF-7 cells, concentrations of RES as low as 0.04 nM up-regulated VDR promoter activity. Maximal induction of VDR promoter activity was observed with 4 nM RES with a magnitude comparable to that obtained with estrogen treatment ([5] and data not shown). Higher concentrations (4 μM) of RES were unable to induce VDR promoter activity in either cell line.

The anti-estrogen TAM was used to determine whether ER was required for VDR promoter up-regulation by 4 nM RES; TAM treatment did not affect VDR promoter activity in T47D cells but blocked the effect of RES, suggesting that RES mediates VDR promoter up-regulation by a process which requires ER (Fig. 2). This suggestion is supported by

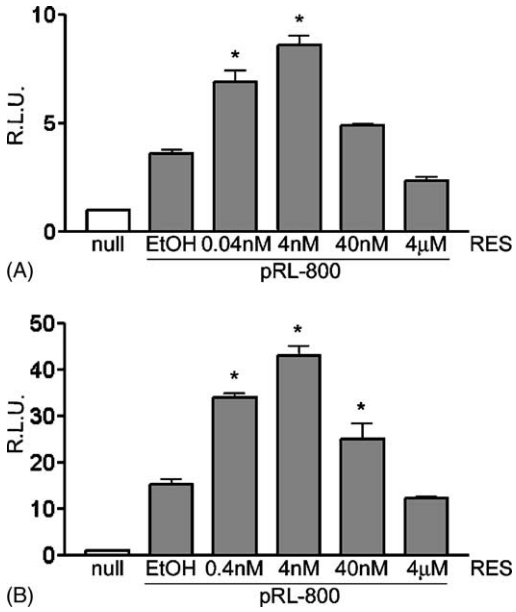

Fig. 1. Effect of resveratrol on human VDR promoter activity in breast cancer cells. T47D (A) and MCF-7 (B) cells were transiently transfected with the pRL-800 human VDR promoter vector or the pRL-null empty vector and treated with 4 nM–4 μM resveratrol (RES) or ethanol (EtOH) vehicle for 18 h. Data are expressed as relative luciferase units (RLU) after correction for transfection efficiency and normalization to pRL-null activity. Mean ± S.E.M. of triplicate transfections are shown; similar results were obtained in three independent experiments, $P < 0.05$, RES treated vs. ethanol control as assessed by one-way ANOVA and Dunnett's post-test.

data that RES did not up-regulate the VDR promoter in an ER negative breast cancer cell line, SUM159-PT (data not shown). Similarly, estrogen did not up-regulate the VDR promoter in MCF-7 and T47D cells in the presence of TAM, or in ER negative SUM159-PT cells ([5] and data not shown).

Parallel studies of T47D and MCF-7 cells transiently transfected with the VDR promoter and treated with

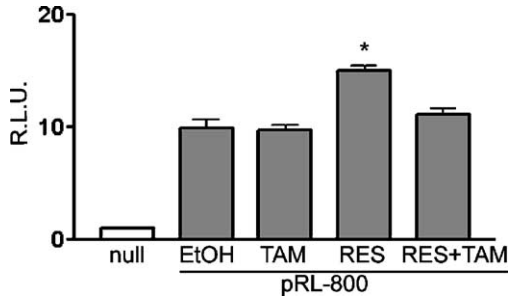

Fig. 2. Effect of tamoxifen on resveratrol induction of human VDR promoter activity. T47D cells were transiently transfected with the pRL-800 human VDR promoter vector and treated with ethanol (EtOH), 100 nM tamoxifen (TAM), 4 nM resveratrol (RES), or RES + TAM for 18 h. Data are expressed as relative luciferase units (RLU) after correction for transfection efficiency and normalization to pRL-null activity. Mean ± S.E.M. of triplicate transfections are shown; similar results were obtained in three independent experiments, $P < 0.05$, RES treated vs. ethanol control as assessed by one-way ANOVA and Dunnett's post-test.

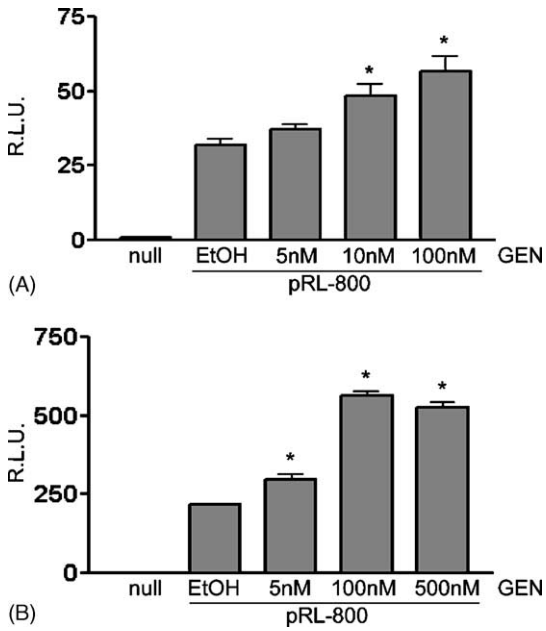

Fig. 3. Effect of genistein on human VDR promoter activity in breast cancer cells. T47D (A) and MCF-7 (B) cells were transfected with the pRL-800 human VDR promoter vector or the pRL-null empty vector and treated with 5–500 nM genistein (GEN) or ethanol (EtOH) vehicle for 18 h. Data are expressed as relative luciferase units (RLU) after correction for transfection efficiency and normalization to pRL-null activity. Mean ± S.E.M. of triplicate transfections are shown; similar results were obtained in three independent experiments, $P < 0.05$, RES treated vs. ethanol control as assessed by one-way ANOVA and Dunnett's post-test.

GEN are presented in Fig. 3. GEN, at concentrations (5–100 nM) that are achievable in vivo after dietary intake, also up-regulated VDR promoter activity in both T47D cells and MCF-7 cells (Fig. 3), but not in the ER negative SUM159-PT cells (not shown).

To determine whether phytoestrogens that up-regulate the VDR promoter activity alter steady state levels of endogenous VDR protein, Western blot analysis of VDR was conducted in T47D cells treated for 48 h with RES or GEN (Fig. 4). An increase in VDR protein expression was observed in cells treated with 4 nM RES (Fig. 4A), the same dose at which maximal up regulation of the VDR promoter

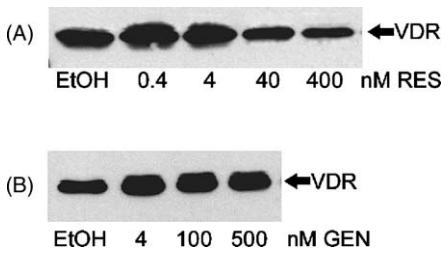

Fig. 4. Effect of phytoestrogens on VDR protein expression in T47D cells. T47D cells were treated for 48 h with (A) 0.4–400 nM resveratrol (RES), (B) with 5–500 nM genistein (GEN) and nuclear extracts isolated as described in Section 2 were separated by SDS–PAGE and blotted for VDR. The blot is representative of three independent experiments that showed similar results.

activity was evident. Higher doses of RES appeared to down regulate VDR protein expression. All doses of GEN tested (5–500 nM) increased VDR protein expression (Fig. 4B). These data show fairly good correlation between regulation of the VDR exon 1c promoter region and endogenous VDR protein expression in response to phytoestrogens in breast cancer cells.

3.2. *Effect of phytoestrogens on growth, cell cycle and ER mediated transcription in breast cancer cells*

Since previous studies described a correlation between VDR expression and proliferation in cultured cells, and estrogen enhances growth of both MCF-7 and T47D cells under the conditions used (data not shown), it was of interest to determine if phytoestrogen regulation of VDR promoter activity or expression was secondary to effects on cell growth. T47D cells were treated for 6 days with RES, GEN or vehicle controls, and growth was assessed by crystal violet assay. As demonstrated in Fig. 5A, GEN stimulated growth of T47D cultures over a broad concentration range (5 nM–1 μM), including doses which up-regulated VDR promoter activity and VDR expression. Low concentrations of RES (0.04–40 nM), the same doses that up-regulated the VDR promoter, did not stimulate growth of T47D cells (Fig. 5B). However, RES at 4 μM significantly increased T47D cell number, and higher concentrations of RES (>20 μM) significantly decreased cell number (Fig. 5B). The growth stimulation induced by 4 μM RES in T47D (Fig. 6A) and MCF-7 (Fig. 6B) cells was blocked by the anti-estrogen tamoxifen, suggesting that the proliferative effect of 4 μM RES is mediated through ER-dependent mechanisms.

Since total cell number reflects both cell proliferation and cell death, the data from the growth assays does not rule out the possibility that low concentrations of RES could alter cell cycle progression. We thus used flow cytometry to determine whether short term treatment of T47D cells with 4 nM or 4 μM RES could mimic the effects of estrogen on the cell cycle. The percentage of cells in G_0/G_1, S and G_2/M phases of the cell cycle after 24 h treatment with ethanol vehicle, 4 nM RES, 4 μM RES or 1 nM estrogen are presented in Table 1. In control (ethanol) treated cultures, the majority (>70%) of cells were in the G_0/G_1 (quiescent) phase, 15% of cells were in S (proliferative) phase, and 10% were in G_2/M (mitosis). The cell cycle profile of cultures treated with 4 nM RES was not different from control cultures. In contrast, cultures treated with 4 μM RES or 1 nM estrogen showed an increased percentage of cells in S phase, and a decreased percentage of cells in G_0/G_1 as compared to control cultures. No differences in the percentages of cells in the G_2/M phase were observed with any treatments. These data are consistent with the crystal violet growth assay results, and confirm that high dose (4 μM) RES mimics the growth stimulatory effects of estrogen, but low dose (4 nM) RES does not promote T47D cell cycle progression.

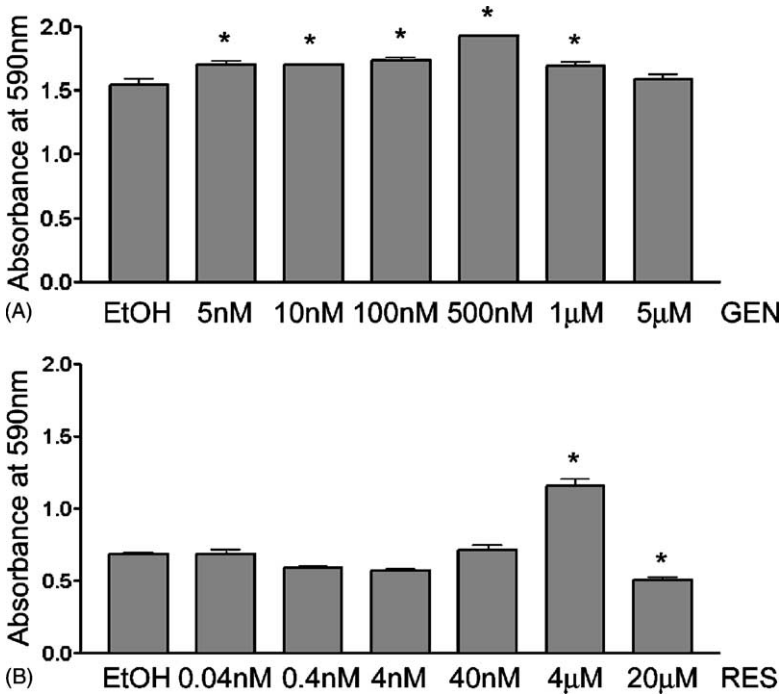

Fig. 5. Effect of phytoestrogens on growth of T47D cells. Cells were treated for 6 days with 5 nM–5 μM genistein (GEN) (A) or 0.04 nM–20 μM resveratrol (RES) (B), or ethanol vehicle (EtOH), and crystal violet growth assays were performed as described in Section 2. Absorbance at 590 nm is proportional to relative cell number. Data are means ± S.E.M. of triplicates; similar results were obtained in three independent experiments, $P < 0.05$, treated vs. ethanol control as assessed by one-way ANOVA and Dunnett's post-test.

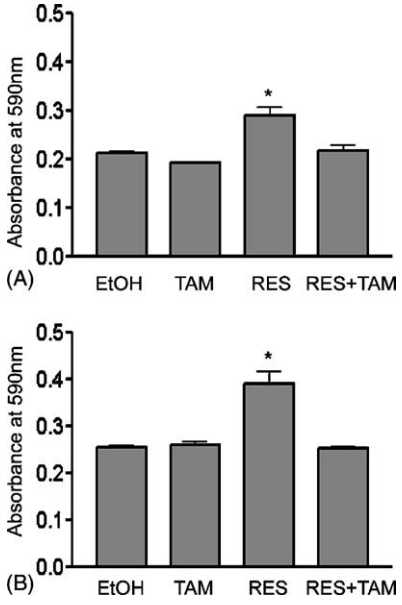

Fig. 6. Effect of tamoxifen on growth stimulation by 4 μM resveratrol. T47D cells (A) or MCF-7 cells (B) were treated with ethanol (EtOH), 100 nM tamoxifen (TAM), 4 μM resveratrol (RES) or RES + TAM for 6 days, and crystal violet assays were performed as described in Section 2. Absorbance at 590 nm is proportional to relative cell number. Data are means ± S.E.M. of triplicates; similar results were obtained in three independent experiments, $P < 0.05$, treated vs. ethanol control as assessed by one-way ANOVA and Tukey's post-test for multiple comparisons.

To assess whether the same concentrations of RES that stimulate proliferation also activate ER mediated gene transcription, T47D cells and MCF-7 cells were transiently transfected with pGL-ERE, an estrogen response element (ERE) luciferase reporter vector, and treated with 1 nM estrogen or 4 μM RES (doses that stimulate cell proliferation in an ER-dependent manner) for 18 h. As shown in Fig. 7A, pGL-ERE activity was up-regulated in T47D cells treated with 1 nM estrogen or with 4 μM RES, and the effect of RES was blocked by the anti-estrogen TAM. Lower concentrations of RES (0.4–40 nM) were unable to induce ERE

Table 1
Cell cycle analysis of T47D cells after 24 h treatment with estrogen or resveratrol

Treatment	G_0/G_1	S	G_2/M
EtOH	73.5 ± 1.6	15.8 ± 0.7	10.7 ± 1.6
E_2 (1 nM)	59.0 ± 0.6*	27.6 ± 0.6*	13.4 ± 2.1
RES (4 nM)	71.7 ± 0.5	14.9 ± 0.8	13.3 ± 2.0
RES (4 nM)	60.9 ± 1.8*	28.6 ± 1.5*	10.5 ± 0.7

T47D cells treated for 24 h with ethanol (EtOH) vehicle, 1 nM estrogen (E_2), 4 nM resveratrol (RES) or 4 μM RES were fixed and incubated with propidium iodide as described in Section 2 for analysis by flow cytometry. Data are the percentage of cells detected in each phase of the cell cycle after modeling DNA histograms with Multiplus software. Mean ± standard error of triplicate samples are shown.

* $P < 0.05$, treated vs. ethanol control as assessed by one-way ANOVA and Dunnett's post-test.

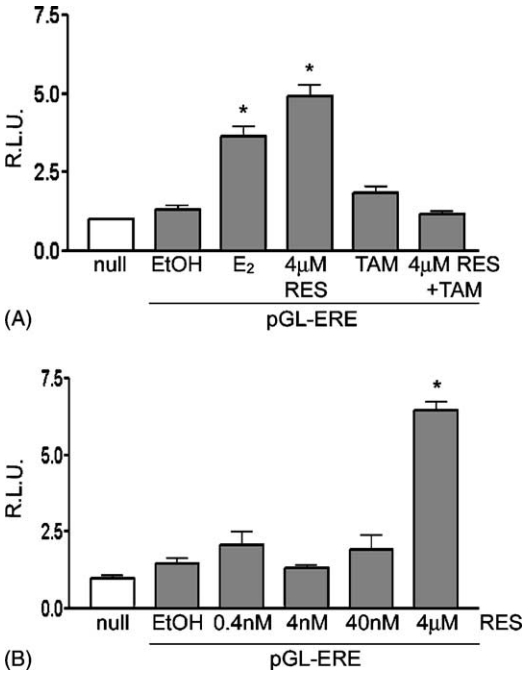

Fig. 7. Effect of resveratrol on ERE-driven luciferase reporter activity in T47D cells. T47D cells were transfected with the pGL-ERE reporter vector and treated with 1 nM estrogen (E_2), 100 nM tamoxifen (TAM), 4 μM resveratrol (RES) or RES + TAM (A), or the indicated doses of RES (B) for 18 h. Data are expressed as relative luciferase units (RLU) after correction for transfection efficiency and normalization to the pGL3-basic empty vector activity. Mean±S.E.M. of triplicate transfections are shown; similar results were obtained in three independent transfection experiments, $P < 0.05$, RES treated vs. ethanol control as assessed by one-way ANOVA and Dunnett's post-test.

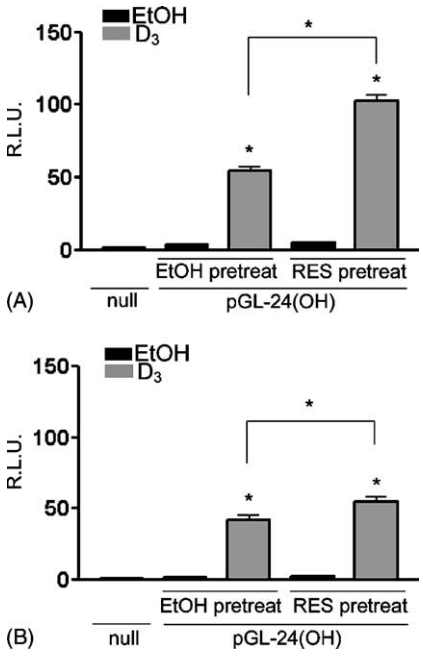

Fig. 8. Effect of resveratrol pre-treatment on VDR mediated transactivation. T47D (A) and MCF-7 (B) cells were pre-treated for 48 h with either ethanol (EtOH) vehicle or 4 nM RES, transiently transfected with the Vitamin D_3 responsive pGL-24(OH) luciferase reporter gene and treated with EtOH or 100 nM $1,25(OH)_2D_3$ (D3) for an additional 18 h. Data are expressed as relative luciferase units (RLU) after correction for transfection efficiency and normalization to the pGL3-basic empty vector activity. Mean ± S.E.M. of triplicate transfections are shown; similar results were obtained in three independent transfection experiments, $P < 0.05$, RES treated vs. ethanol control, other statistically significant comparisons are indicated with solid lines. Significance was assessed by one-way ANOVA and Tukey's post-test.

reporter activity in T47D cells (Fig. 7B). Similar results for RES regulation of the pGL-ERE luciferase reporter gene were obtained in MCF-7 cells (not shown). These data show that 4 μM RES, a concentration that stimulates growth of breast cancer cells, also up-regulates ERE mediated transcription. However, lower doses of RES (4 nM), which up-regulate the VDR promoter, do not stimulate growth or activate ERE mediated transcription.

3.3. Effect of phytoestrogens on cellular sensitivity to 1,25(OH)$_2$D$_3$

Because RES up-regulates VDR at doses that do not enhance breast cancer cell proliferation, we hypothesized that this phytoestrogen might exert beneficial effects against cancer by sensitizing cells to the anti-proliferative effects of $1,25(OH)_2D_3$. To determine cellular sensitivity to $1,25(OH)_2D_3$, we assessed VDR mediated transactivation in cells transiently transfected with pGL-24(OH), a reporter vector that contains the $25(OH)D_3$-24-hydroxylase promoter. This promoter contains two Vitamin D_3 response elements and is highly induced by $1,25(OH)_2D_3$ in cells that express VDR. To examine whether RES could sensitize cells to $1,25(OH)_2D_3$, T47D and MCF-7 cells were pre-treated with 4 nM RES or ethanol vehicle for 48 h, then challenged with 100 nM $1,25(OH)_2D_3$ or ethanol vehicle. In cells pre-treated with ethanol vehicle, 18 h treatment with 100 nM $1,25(OH)_2D_3$ increased pGL-24(OH) reporter activity approximately 50-fold in T47D (Fig. 8A) and MCF-7 (Fig. 8B) cells. Pre-treatment with 4 nM RES for 48 h did not alter basal activity of the pGL-24(OH) reporter, but in both T47D and MCF-7 cells, 24-hydroxylase reporter activity in response to $1,25(OH)_2D_3$ was significantly enhanced.

Since low doses of RES that do not stimulate proliferation are able to enhance $1,25(OH)_2D_3$ inducible VDR transcriptional activity, we hypothesized that RES would sensitize cells to the anti-proliferative effects of $1,25(OH)_2D_3$. To test this hypothesis, growth was assessed in T47D cells treated for 8 days with 100 nM $1,25(OH)_2D_3$ (a dose that causes maximal growth inhibition) in the presence or absence of 4 nM RES (a dose that up-regulates VDR expression and transactivation). Cultures were treated in parallel with 100 nM $1,25(OH)_2D_3$ in the presence or absence of 1 nM estrogen, a dose that also up-regulates VDR expression and transactivation [5,16]. As shown in Fig. 9A, RES alone had no effect on T47D cell growth, but the combination treatment of RES and $1,25(OH)_2D_3$ decreased cell growth

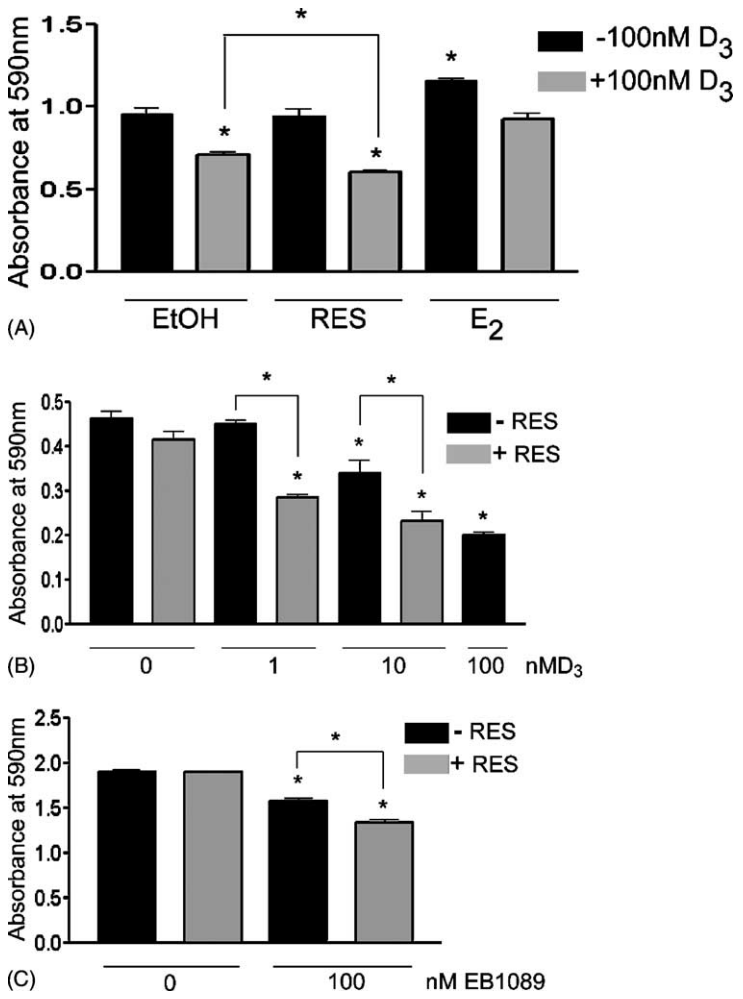

Fig. 9. Effect of resveratrol pre-treatment on sensitivity of T47D cells to VDR mediated growth inhibition. (A) T47D cells were pre-treated with ethanol (EtOH) vehicle, 4 nM resveratrol (RES), or 1 nM estrogen (E_2), then treated with 100 nM $1,25(OH)_2D_3$ in the continued presence or absence of RES or E_2 for 8 days with two media changes. (B) T47D cells were pre-treated with EtOH or 4 nM RES then treated with varying concentrations of $1,25(OH)_2D_3$ (D3) as indicated, in the presence or absence of RES, for additional 5 days with one media change. (C) T47D cells were pre-treated with EtOH vehicle or 4 nM RES, then treated with 100 nM EB1089 for 8 days in the presence or absence of RES. Media and treatments were replaced three times. Crystal violet growth assays were performed as described in Section 2. Absorbance at 590 nm is proportional to relative cell number. Data are means ± S.E.M. of triplicates; similar results were obtained in three independent experiments, $P < 0.05$, treated vs. ethanol control, other statistically significant comparisons are indicated with solid lines. Significance was assessed by one-way ANOVA and Tukey's post-test.

more than $1,25(OH)_2D_3$ alone. In contrast, estrogen alone increased T47D cell growth and counteracted the growth inhibitory effects of $1,25(OH)_2D_3$ under the same conditions.

To test whether RES could enhance cellular sensitivity to lower doses of $1,25(OH)_2D_3$, T47D cells were treated for 5 days with $1,25(OH)_2D_3$ in the presence or absence of 4 nM RES (Fig. 9B). At 1 nM, $1,25(OH)_2D_3$ alone did not reduce cell growth, however combination treatment of RES with 1 nM $1,25(OH)_2D_3$ reduced cell number by approximately 40%. Similarly, 10 nM $1,25(OH)_2D_3$ reduced cell number by 25% in the absence of RES and 50% in the presence of RES. In the presence of RES, 10 nM $1,25(OH)_2D_3$ was as effective in growth inhibition as 100 nM $1,25(OH)_2D_3$. Thus, RES alone did not affect growth of T47D cells, co-treatment with RES enhanced cellular response to the growth inhibitory effects of $1,25(OH)_2D_3$. To examine whether a similar sensitization could be achieved with a synthetic Vitamin D_3 analog, growth of T47D cells was assessed after treatment with 100 nM EB1089 in the presence or absence of 4 nM RES (Fig. 9C). As expected, T47D cells were growth inhibited by 100 nM EB1089, and as predicted, combination treatment with EB1089 and RES was more effective than EB1089 alone.

4. Discussion

In previous studies, we demonstrated that estrogen enhances VDR promoter activity and transactivation by $1,25(OH)_2D_3$ in breast cancer cells [5]; however, estrogen simultaneously stimulates breast cancer cell proliferation and counteracts the growth inhibitory effects of

$1,25(OH)_2D_3$. We therefore sought to identify additional estrogen-like compounds that could selectively mimic the ability of estrogen to up-regulate VDR signaling without enhancing cell proliferation. In theory, such compounds might enhance the growth inhibitory effects of $1,25(OH)_2D_3$ and Vitamin D analogs on breast cancer cells. The present studies were designed to test the hypothesis that phytoestrogens could sensitize breast cancer cells to the anti-proliferative effects of $1,25(OH)_2D_3$ via up regulation of the VDR. A major outcome of our work is the identification of RES as a naturally occurring estrogen-like compound which enhances $1,25(OH)_2D_3$ mediated growth inhibition of T47D and MCF-7 cells. At doses which do not increase proliferation, RES up-regulates the human VDR gene promoter, increases VDR protein expression, enhances VDR transactivation ability and sensitizes breast cancer cells to the anti-proliferative effects of $1,25(OH)_2D_3$. These are the first studies to demonstrate effects of low (nM) concentrations of RES (which are representative of circulating concentrations of this phytoestrogen after dietary intake [10]) on breast cancer cells. Although the phytoestrogen GEN also up-regulates the human VDR promoter and VDR protein expression, GEN stimulates proliferation of breast cancer cells over a wide dose range, and therefore is not a suitable candidate for breast cancer treatment or prevention. Although both RES and GEN mimic some actions of estrogens, notable differences in their ultimate biological effects on breast cancer cells exist.

RES is a polyphenol present in grapes, nuts and red wine that has been shown to bind ER and activate a variety of estrogen responsive target genes in breast cancer cells [17]. The low binding affinity of RES for ER suggests that high concentrations of RES are required for estrogenic activity [13,17], and in these studies we confirm that μM concentrations of RES are required to activate an ERE-driven luciferase reporter gene in breast cancer cells. However, we also show that doses of RES in the nM range mimic estrogen regulation of the VDR promoter. RES mediated enhancement of VDR promoter activity was blocked by the anti-estrogen TAM and did not occur in ER negative breast cancer cells, suggesting that RES, like estrogen [5], regulates the VDR promoter through an ER-dependent mechanism. However, since the doses of RES that activate the VDR promoter do not activate an ERE-driven luciferase reporter gene, and there are no consensus ERE sequences in the VDR promoter [5], RES regulation of the VDR is likely mediated through ER-dependent activation of alternative transcription factors such as Sp1 or AP1. Several estrogen responsive genes, such as bcl-2, have been identified which lack ERE sites but are regulated by estrogen, in an ER-dependent manner, through AP1 or Sp1 sites [18,19] and we have identified several potential Sp1 and AP1 sites in the VDR promoter sequence [5]. We are therefore testing the hypothesis that VDR promoter regulation by estrogens and phytoestrogens, including RES and GEN, is mediated through ER interaction with additional transcription factors such as Sp1 and AP1. At present, we cannot speculate whether these effects are mediated via ERα, ERβ or both, since the cell lines utilized in the present study express both ER subtypes [15].

One caveat to our studies, however, is our demonstration that at 4 μM, RES stimulates breast cancer cell proliferation and cell cycle progression comparably to estrogen. The proliferative effect of RES is only observed at low μM concentrations, as we and others [11,12] have observed that doses of 20 μM or higher RES markedly reduce cell growth and induce apoptosis. While μM doses of RES are not likely to be achieved after consumption of RES from dietary sources, the circulating concentration of RES after ingestion of purified RES supplements has yet to be determined. Thus it is not clear if RES concentrations in the μM range are achieved in vivo, and if so, whether these doses would exert stimulatory or inhibitory effects on mammary cell proliferation in vivo.

In summary, these are the first studies to demonstrate interactions between phytoestrogens and the Vitamin D_3 endocrine system in regulation of breast cancer cell growth. Enhancement of Vitamin D_3 signaling by RES occurs at doses in the nM range, and these concentrations are similar to those which are achieved in serum after dietary ingestion [10]. Our data support the novel concept that dietary consumption of RES may increase VDR expression and sensitize breast cancer cells to the anti-proliferative effects of $1,25(OH)_2D_3$. The significance of these findings relates to the potential therapeutic applications of Vitamin D_3 analogs in breast cancer patients, since a patient's response to Vitamin D_3 analog treatment could be limited by the level of VDR expression. Our results offer proof of principle that phytochemicals can impact on cellular sensitivity to $1,25(OH)_2D_3$, and thus suggest that dietary modifications may enhance the therapeutic efficacy of Vitamin D_3 analogs. However, further studies to extend our in vitro findings to an in vivo model are necessary in order to clarify the potential risks and benefits of phytoestrogen ingestion and to assist in generation of rational dietary recommendations. On a more general note, these studies show that complex molecular interactions between estrogens, including phytoestrogens, and $1,25(OH)_2D_3$ occur through their cognate nuclear receptors in breast cancer cells.

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