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**VALPROIC ACID RESTORES ER α AND ANTIESTROGEN SENSITIVITY
TO ER α -NEGATIVE BREAST CANCER CELLS.**

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ABSTRACT**Key Words:** breast cancer, ER α , HDI, VPA, antiestrogen

Histone deacetylase inhibitors (HDIs) are valuable drugs in breast cancer where estrogen receptor α (ER α) can be silenced by epigenetic modifications. We report the effect of the clinically available HDI, valproic acid (VPA), on ER α expression and function in ER-negative breast cancer cells, MDA-MB-231. VPA induced ER α mRNA and protein, while did not modify ER β . In VPA-treated cells, we also observed: 1) a correct transcriptional response to estradiol after transfection with the luciferase gene under the control of an estrogen-responsive minimal promoter (ERE-TKluc); 2) increased expression of the ER-related transcription factor FoxA1; 3) estradiol-induced up-regulation of several estrogen-regulated genes (e.g. pS2, progesterone receptor); 4) inhibitory effect of tamoxifen on cell growth. In conclusion, the HDI VPA, inducing ER α and FoxA1, confers to MDA-MB 231 cells an estrogen-sensitive “phenotype”, restoring their sensitivity to anti-estrogen therapy.

INTRODUCTION

The expression of estrogen receptor α (ER α) in breast cancer is the necessary molecular requisite for treatment with the antiestrogen tamoxifen (Osborne, 1998; Katzenellenbogen and Frasor, 2004). Unfortunately, a portion of breast cancer is ER α -negative at the time of diagnosis, and an additional portion of tumours loses receptor expression as disease progresses. The absence/loss of ER α has been associated to insensitivity to antiestrogen therapy (Clarke et al., 2001; Ring and Dowsett, 2004). However, the simple re-expression of ER α is not sufficient to restore the estrogen-sensitivity of cells; it is now recognized that the function of ER largely depends on other transcription factors, that are expressed in a tissue-specific way and that can in turn be affected by the state of chromatin (Kininis and Kraus, 2008). One of the possible mechanisms for ER α loss of expression is silencing of the gene by epigenetic modification such as histone tail deacetylation and methylation, and methylation of the CpG islands in the ER promoter (Ottaviano et al., 1994; Lapidus et al., 1998). Therefore, one of the new therapeutic strategies that have been currently receiving much attention is based on the use of agents interfering with chromatin remodelling process. Both demethylating agents like 5-aza-2'-deoxy-cytidine and histone deacetylase inhibitors (HDIs) were shown to reactivate ER α expression in ER-negative breast cancer cells, especially when used in association (Yang et al. 2000; Yang et al., 2001; Keen et al., 2003; Sharma et al., 2006). More interesting, HDIs were reported to induce also the expression of progesterone receptor mRNA (Keen et al., 2003) and a new HDI, LBH589, was shown to induce ER α in MDA-MB-231 cells (Zhou et al., 2007). Among transcription factors coadjuvating ER, FoxA1 has received much attention in breast cancer. It helps the recruitment of ER α on chromatin (Robyr et al., 2000) and it is required for optimum expression of almost 50% of estrogen-regulated genes (Carroll et

al., 2005; Laganière et al., 2005; Carroll et al., 2006). Also FoxA1 function depends on histone modifications and has been demonstrated that its binding to chromatin translates an epigenetic signature into cell type-specific transcriptional programs (Lupien et al., 2008).

We reported elsewhere that valproic acid (VPA) is a powerful antiproliferative agent in estrogen-sensitive cells (Fortunati et al., 2008) and this drug was demonstrated to enhance the efficacy of the antiestrogen tamoxifen through increasing ER-mediated transcription (Hodges-Gallagher et al., 2007). Furthermore, VPA, a potent anticonvulsant that acts also as a class I selective HDAC inhibitor (Gottlicher et al., 2001; Phiel et al., 2001), produces mild adverse effects in man, even when serum levels exceed the normal therapeutic range while receiving antiepileptic therapy (Chapman et al., 1982).

The aim of the present study was to evaluate the effect of VPA on the expression of ER α in the ER-negative breast cancer cell line MDA-MB-231 and its capability to restore the full function of the newly-expressed receptor. The ultimate goal was to define the ability of VPA to confer to breast cancer cells anti-estrogen sensitivity in order to explore new therapeutic options in estrogen-insensitive breast tumours.

MATERIALS AND METHODS

Cell lines and culture conditions

Human estrogen receptor-negative breast cancer MDA-MB-231 cells and human estrogen-dependent MCF-7 breast cancer cells used as positive control were purchased from ECACC (Salisbury, UK).

MCF-7 and MDA-MB-231 breast cancer cells were routinely maintained in RPMI 1640 (Sigma, St Louis, MO, USA) supplemented with 10% heat inactivated FBS (Euroclone, Wetherby, West York, UK). Before experiments, cells were seeded in modified RPMI 1640 (Sigma, St Louis, MO, USA) supplemented with 10% FBS and specifically treated with dextran-coated charcoal to remove steroids. After 48 hours, cells were treated with VPA (0.5-1.5 mM) for further 48 hours, followed, where indicated, by a 72 hour hormonal treatment (10 nM estradiol and/or 1 μ M 4-OH-tamoxifen).

Gene expression evaluation with Real Time PCR

RNA extraction

Cells (2×10^5 cell/well) were seeded in 6-well plates and treated as described above. Total RNA was extracted using TRIzol Reagent (Invitrogen Ltd, Paisley, UK), as previously described. DNase I was added to remove remaining genomic DNA.

cDNA synthesis and PCR amplification- End Point-RT PCR

Total RNA was reverse-transcribed at 42° C for 40 min using reverse transcriptase and oligodT primer (Invitrogen, Groningen, The Netherlands). The PCR reaction system

contained 5 μ l of 10X PCR buffer, 10 μ l of RT product, 0.2 mM dNTP (Invitrogen, Groningen, The Netherlands), 1.25U Taq DNA polymerase (Biorad Laboratories, Inc). ER α primers: forward 5'- CAT AAC GAC TAT ATG TGT CCA GCC and reverse 5'- AAC CGA GAT GAT GTA GCC AGC AGC; ER β primers: forward 5'- TTC CCA GCA ATG TCA CTA ACT, reverse 5'- TCT CTG TCT CCG CAC AAG G; FOXA1 primers: forward 5'-GGG TGG CTC CAG GAT GTT AGG, reverse 5'- GGG TCA TGT TGC CGC TCG TAG; β actin primers: forward 5'- CTC ACC CTG AAG TAC CCC ATC G and reverse 5'- CTT GCT GAT CCA CAT CTG CTG G. Amplification was carried out as follows: 1 X (95°C, 2 min); 35 X (95°C, 30 s; 65°C, 30 s; 72°C, 30 s); and 1 X (72°C, 7 min) for ER α and β actin; 1 X (95°C, 3 min); 40 X (95°C, 30 s; 50°C, 1 min; 72°C, 2 min); and 1 X (72°C, 7 min) for ER β and 1 X (95°C, 5 min); 40 X (95°C, 15 sec, 60°C, 30 sec) for FoxA1. PCR products were electrophoresed on 1.5% agarose gel in the presence of ethidium bromide. Gels were photographed and analyzed with Kodak 1D Image Analysis software.

cDNA synthesis and PCR amplification- RealTime PCR

1 μ g of total RNA was reverse-transcribed with iScript cDNA Synthesis Kit (BioRad Laboratories, Inc.), following manufacturer's protocol.

Primers were designed using Beacon Designer 5.0 software according to parameters outlined in the BioRad iCycler Manual. Specificity of primers was confirmed by BLAST analysis. ER α primers used were as follows: forward 5'-TGT GTC CAG CCA CCA ACC AG, reverse 5'-TTC AAC ATT CTC CCT CCT CTT CGG. PR primers: forward 5'-GCA TCA GGC TGT CAT TAT GGT GTC, reverse 5'-CAT AAG TAG TTG TGC TGC CCT TCC. ADBR1 primers: forward 5'-AGC CCA CAA TCC TCG

TCT GAA TC, reverse 5'-GCA AGC CAC TCT CCC ATC CC. AMFR primers: forward 5'-TTC CTA CAC AGC GGT CAG ATA GC, reverse 5'-GCC GAA GTC CAG CGT CTC C. pS2 primers: forward 5'-CAG CAC GGT GAT TAG TCC CAG AG, reverse 5'-AGA AGC GTG TCT GAG GTG TCC. AREG primers: forward 5'-TGG TGC TGT CGC TCT TGA TAC TC, reverse 5'-CGA GGA CGG TTC ACT ACT AGA AGG. FoxA1 primers: forward 5'-GGG TGG CTC CAG GAT GTT AGG, reverse 5'-GGG TCA TGT TGC CGC TCG TAG; β -actin primers: forward 5'-GCG AGA AGA TGA CCC AGA TC, reverse 5'-GGA TAG CAC AGC CTG GAT AG.

Real-time PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories, Inc.) with SYBR green fluorophore. Reactions were performed in a total volume of 25 μ l-included 12.5 μ l IQ SYBR Green Supermix (BioRad Laboratories, Inc.), 1 μ l of each primer at 10 μ M concentration, and 5 μ l of the previously reverse-transcribed cDNA template. Protocol for primer set was optimised using seven serial 5X dilutions of template cDNA obtained from MCF-7 in basal conditions. The protocol used is as follows: denaturation (95°C for 5 min), amplification repeated 40 times (95°C for 15 sec, 60°C for 1 min). A melting curve analysis was performed following every run to ensure a single amplified product for every reaction. All reactions were carried out at least in triplicate for each sample.

Luciferase Transcription Assay

MDA-MB-231 and MCF-7 cells (2.5×10^5) were seeded in 6-well plates. 24 hours after seeding, cells were treated with 0.7 mM VPA, and 24 hours later, were transfected with 1 μ g of ERE-TKluc plasmid (Cicatiello et al., 2004) (a kind gift of Prof. A.Weisz, Dipartimento di Patologia Generale, Seconda Università degli Studi di Napoli) using

Lipofectin Reagent (Invitrogen Ltd, Paisley, UK). After 24 hours, cells were treated with 10 nM estradiol for further 24 hours. At completion, luciferase activity was assayed with the Luciferase Assay System (Promega Corp.).

ER α Immunofluorescence microscopy

MDA-MB-231 and MCF-7 cells (3×10^5) were seeded in 6 cm dishes. After 48 hours, MDA-MB-231 cells were treated with 0.7 mM VPA for 72 hours. Then cells were fixed in acetone/methanol (1:1) at -20°C for 20 minutes, washed with PBS containing 0.5% Triton X-100, 0.05% NaN_3 and 1% Horse Serum and incubated with polyclonal anti-ER α antibody (1:100), (Santa Cruz Biotechnology, inc., California, USA) in PBS at 4°C overnight. Then, cells were washed with PBS containing 0.5% Triton and 0.05% NaN_3 for 10 minutes for three times followed by detection with anti-rabbit Cy3-conjugated secondary antibody (1:1000), (GE Healthcare Europe, GmbH, Milan, Italy) in PBS plus 0.5% Triton and 0.05% NaN_3 for 2 hours and nuclei counterstaining with Hoechst 33258 (500 ng/ml in DMSO) in PBS. Cells were washed twice with distilled water and mounted with 50% glycerol-PBS media.

Assessment of cell number

Cells were seeded at 3×10^3 cells/well in 96-well plates (Corning, New York, NY, USA). After treatments, cell number was assessed with 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulphonate (WST-1, Roche, Penzberg Germany) incorporation. Briefly, 10 μl of WST-1 were added to each well; after 1 hour incubation, absorbance at 450 nm was measured using a plate reader (Model 680 Microplate Reader, Bio-Rad, Hercules, CA, USA). Four replicates were done to determine each data point.

Statistical analysis

Data are expressed throughout the text as mean \pm SEM, calculated from at least three different experiments. Statistical comparison between groups was performed with one way analysis of variance (ANOVA) and the threshold of significance was calculated with the Bonferroni test. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of VPA on ER α

ER α was evaluated both as mRNA and protein expression.

MDA-MB-231 cell did not express any mRNA for ER α , in basal condition, but after treatment with 0.7 mM VPA for 72 hours, it was easily detectable. In MCF-7 cells, that express ER α in basal conditions, VPA did not alter the receptor level of expression. On the contrary, VPA had no effect on the expression of ER β in both cell lines (Figure 1). Results and dose-response curve were further confirmed by Real Time PCR (Figure 1, lower panel).

As shown in Figure 2, immunofluorescence for ER α was positive in MCF-7 (panel A), but not in MDA-MB-231 (panel B) in basal conditions; after VPA treatment, immunofluorescence for ER α became positive also in MDA-MB-231 cells (panel C).

Effect of VPA on Cell Transcriptional Response to Estradiol

Figure 3 reports the transcriptional response to estradiol of MCF-7 and MDA-MB-231 cells. After transient transfection with a reporter plasmid carrying the luciferase gene under the control of an estrogen-responsive minimal promoter (ERE-TKluc), MCF-7 cells showed a significant induction of transcription after estradiol treatment ($p < 0.001$) without any modification induced by VPA. Transfected MDA-MB-231 cells that did respond neither to estradiol nor to VPA, when used alone, presented a significant increase in transcriptional activity ($p < 0.001$) when estradiol treatment was performed after VPA exposure.

Effect of VPA on FoxA1 Expression

The transcription factor FoxA1, functionally linked to ER α , was expressed in basal condition in MDA-MB-231 cells and in ER-positive MCF-7 cells; VPA treatment significantly increased FoxA1 level in MDA-MB-231 cells. No modification of FoxA1 basal level was observed in MCF-7 cells after VPA treatment (Figure 4).

Effect of VPA and estradiol on estrogen-regulated genes

In order to determine whether the re-expressed ER α is sufficient to mediate gene activation in response to estradiol, a number of estrogen-regulated genes were selected and analyzed by qRT-PCR. PR and pS2 are well-known estrogen-induced genes in MCF-7 cells, whereas AREG, ADRB and AMFR were selected from a database obtained by microarray analysis (Cicatiello et al., 2004; Scafoglio et al., 2006).

Detectable levels of mRNA of these genes were expressed in MDA-MB-231 cells, and none of them was influenced by either VPA or estradiol treatment. However, after VPA treatment, estradiol was able to induce expression of all of them, significantly increasing their mRNA level (Figure 5).

Effect of VPA on Cells Proliferative Reponse to Estradiol and Tamoxifen

As shown in Figure 6, MDA-MB-231 cells did not modify their growth after exposure to estradiol and 4OH-tamoxifen. After treatment with 0.7 mM VPA MDA-MB-231 cells assumed a behavior similar to that of MCF-7 cells, increasing their growth under estradiol treatment and, especially, reducing it under tamoxifen. VPA did not modify MCF-7 cells response to either estradiol and tamoxifen.

DISCUSSION

The present study describes in details the ability of VPA to restore an estrogen-sensitive phenotype in ER α -negative breast cancer cells, allowing successful treatment with the anti-estrogen tamoxifen *in vitro*.

ER α is one of the key molecules for the treatment of breast cancer. The absence/loss of ER α expression is linked to insensitivity to antiestrogen therapy (Clarke et al., 2001; Ring et al., 2004) and the possibility to sensitize these tumors to tamoxifen by inducing ER α expression with HDIs and/or demethylating agents is at present one of the goals of the novel treatments.

We show here that VPA induces re-expression of ER α gene and protein in ER-negative cell line, MDA-MB-231. After VPA treatment, the localization of ER is prevalently cytoplasmatic; however, our observation about the restored function of ER suggests the nuclear translocation of the receptor after estradiol treatment, a condition necessary for its transcriptional activity. It is important to note that the mRNA for ER α is detectable already at 0.7 mM VPA, a dose that is reported to be free of significant toxic effects in man (Gottlicher et al., 2001). It was recently reported (Biçaku et al., 2008) that VPA had no effect on ER α expression in MDA-MB-231 cells and authors claimed that it was due to the lack of inhibition of HDAC6 exerted by VPA. Actually, we reported elsewhere that VPA is able to inhibit HDAC6 activity (Catalano et al., 2007), in agreement with others (Gottlicher et al., 2001). Moreover, very high doses of VPA (2 mM), as those used by Biçaku and coworkers, are likely to be toxic. In effect, we observed that ER α expression was dose-dependent, reaching a maximum at 1.5 mM VPA; treatment with doses > 1.5 mM had a clear toxic effect in cells and, therefore, receptor determination was no longer possible and reliable (unpublished data). Thus, the therapeutic levels of VPA reached in patients treated for epilepsy (0.3-1 mM) are adequate to induce ER α expression. Furthermore, this dose is clinically safe and has no

severe side-effects, since only limited toxicity occurs when the concentration is below 3.1 mM, and severe side effects develop when the concentration is above 5.9 mM (DeVane 2003).

The re-expression of silenced genes, to be biologically relevant, has to be followed by the appearance of their function. We show that the receptor expressed in MDA-MB-231 cells after VPA treatment was perfectly functional as transcriptional factor, as demonstrated by luciferase experiment and induction of estrogen-regulated genes. VPA alone has no effect on PR mRNA expression (as well as on the mRNA expression of the other estrogen-regulated genes), but all genes were significantly up-regulated by estradiol after VPA treatment. This observation is in line with data on receptor expression and luc-trascription induction, pointing out that VPA treatment of ER-negative cells is capable to sensitize cells to estradiol effect and this is due to the expression of a functional ER α . Even though going the opposite, data from De Los Santos and coworker (2007) demonstrated the same mechanism of action. In fact, in MCF-7 cells butyrate, after having down-regulated ER α , inhibited estradiol-induced PR and pS2 expression.

In addition, we show for the first time that VPA increases also the level of expression of the transcription factor FoxA1 and this observation is of particular relevance in the evaluation of the integrity of ER-pathway. FoxA1 is a transcription factor whose expression correlates with the presence of ER α in breast tumours. Knocking-down FoxA1 expression in MCF-7 cells causes the reduction of estrogen-regulated gene expression and cell growth (Carroll et al., 2006; Laganière et al., 2005). FoxA1 helps the recruitment of ER α on chromatin (Robyr et al., 2000) and is required for optimum expression of almost 50% of estrogen-regulated genes (Carroll et al., 2005; Laganière et al., 2005; Carroll et al., 2006). Furthermore, FoxA1 expression in breast cancer patients

is associated with good prognosis (Badve et al., 2007; Thorat et al., 2008) and it is a marker of luminal subtype (Yamaguchi et al., 2008). It is thus likely that FoxA1 delineates a further level of estrogen-sensitivity of breast cancer cells. The ability of VPA to increase the level of expression of FoxA1 in MDA-MB-231 cells, as well, gives even more strength to its ability of restoring estrogen-sensitivity. Acting on both ER α and FoxA1, VPA not only restores a functioning receptor in MDA-MB 231 cells but also implements the molecular pathway used by ER to obtain estrogen ultimate effects in breast cancer cells.

Finally, we clearly demonstrated that VPA-induced ER α , through estradiol-regulated transcription and gene expression, confers to MDA-MB-231 cells estrogen sensitivity. In fact, cells, after VPA treatment, modify their growth pattern increasing it when treated with estradiol, but, especially, they acquired a correct growth response to the anti-estrogen tamoxifen.

In conclusion, we suggest that HDI treatment of ER-negative breast tumours could be a useful tool to gain anti-estrogen sensitivity, and a careful analysis of the integrity of the estrogen-sensitive pathway should be evaluated in each single patient before the use of combined HDI/antiestrogen schedules.

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LEGEND TO FIGURES

Figure 1 – Effect of VPA on ER α and ER β gene expression. End Point RT-PCR was performed on RNA obtained from MDA-MB-231 and MCF-7 breast cancer cells in basal condition and after treatment with 0.7 mM VPA; β -actin was also evaluated as house-keeping gene. Real-time PCR for ER α on RNA from MDA-MB-231 after treatment with increasing doses of VPA (0.5-1mM) (*lower panel*)..

Figure 2 - Effect of VPA on ER α protein expression. Immunofluorescence for ER α was performed on MDA-MB-231 cells in basal condition (B) and after 0.7 mM VPA treatment (C); MCF-7 cells in basal condition were used as positive control (A).

Figure 3 – Effect of VPA on transcriptional response to estradiol. MDA-MB 231 and MCF-7 breast cancer cells were transiently transfected with a reporter plasmid carrying the luciferase gene under the control of an estrogen responsive minimal promoter (ERE-TKluc). The luciferase activity was assayed before and after 10 nM estradiol treatment. Data are expressed as mean \pm SD obtained from three different experiments.

Figure 4 – Effect of VPA on FoxA1 expression. End Point and Real Time RT-PCR for FoxA1 was performed on RNA obtained from MDA-MB-231 and MCF-7 cells in basal conditions and after 0.7 mM VPA treatment. β -actin was also evaluated as house-keeping gene. Results were normalized for the house keeping gene (β -actin) parameters and expressed as $E_{ref}^{C_{tref}}/E_{tar}^{C_{ttar}}$, where E_{ref} is PCR efficiency and C_{tref} is threshold

cycle for the house keeping gene while E_{tar} and C_{tar} are the same parameters for the target gene. Significance: **, $P < 0.01$.

Figure 5 – Effect of VPA on estrogen-regulated genes in MDA-MB-231 cells. The estrogen-regulated genes PR, pS2, AREG, ADRB, AMFR were evaluated with Real Time PCR in cells treated with 10 nM estradiol, in the absence or in the presence of 0.7 mM VPA. Results were normalized for the house keeping gene (β -actin) parameters and expressed as $E_{ref}^{C_{ref}}/E_{tar}^{C_{tar}}$, where E_{ref} is PCR efficiency and C_{ref} is threshold cycle for the house keeping gene while E_{tar} and C_{tar} are the same parameters for the target gene. NS, not significant; *, $P < 0.05$; **, $P < 0.01$.

Figure 6 –Effect of VPA on proliferative response to estradiol and tamoxifen. Relative number of cells was assessed with WST-1 in MDA-MB-231 and MCF-7 breast cancer cells after treatment with 10 nM estradiol (E_2), 10 nM 4OH-tamoxifen (TAM) or both (E_2 +TAM), either in basal condition or after 0.7 mM VPA treatment. NS, not significant; ***, $P < 0.001$.

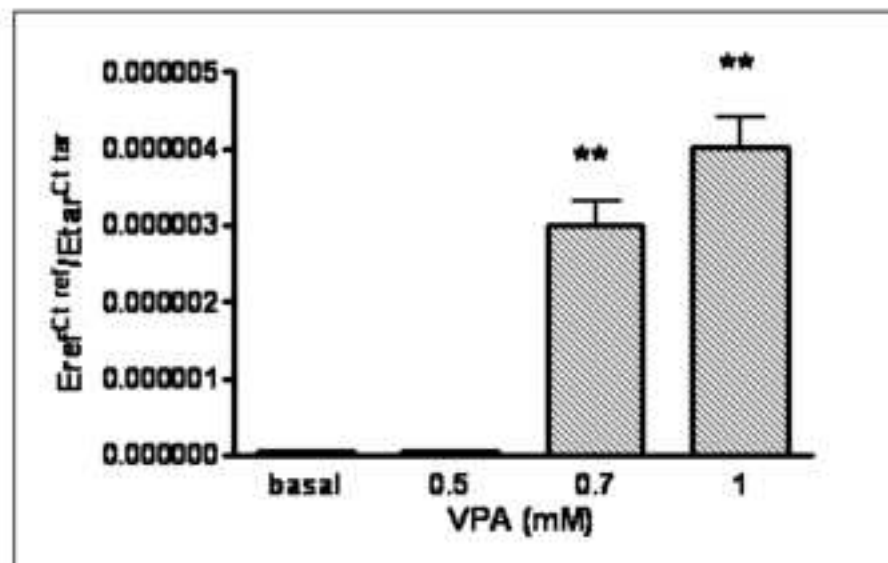
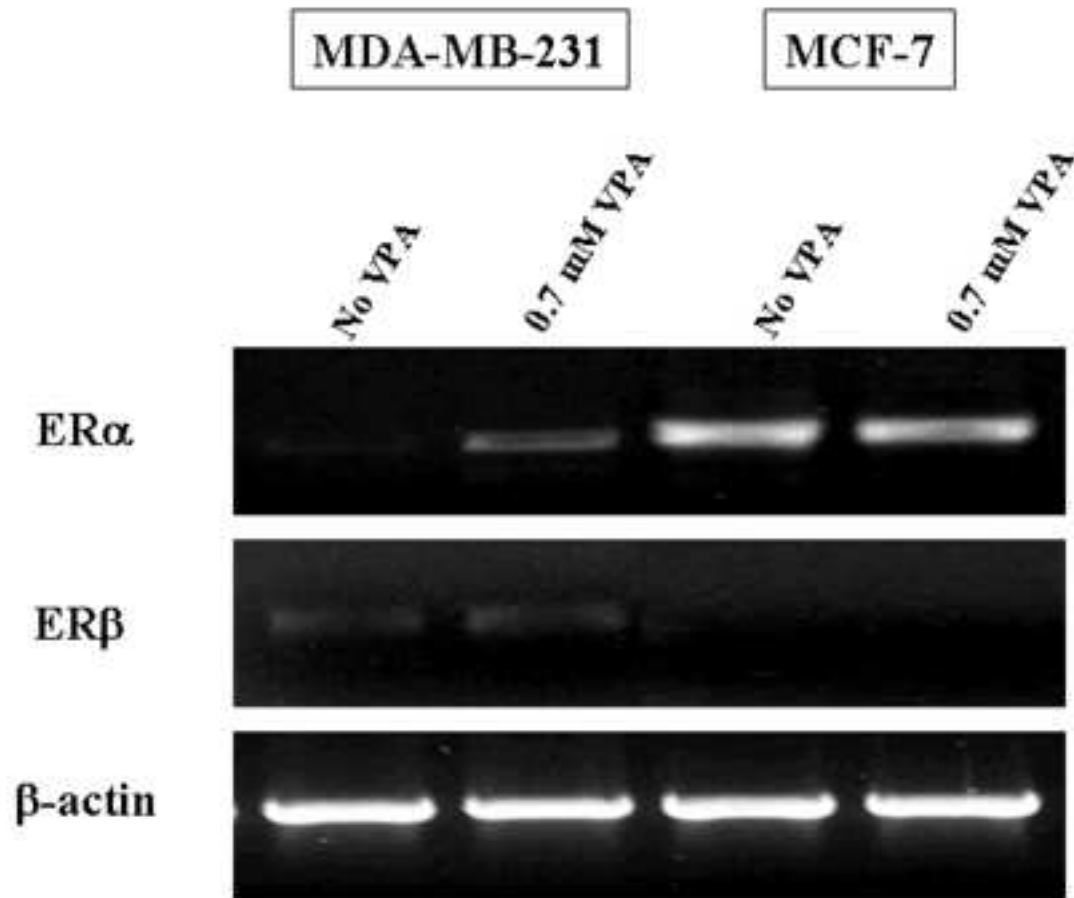


Figure 1

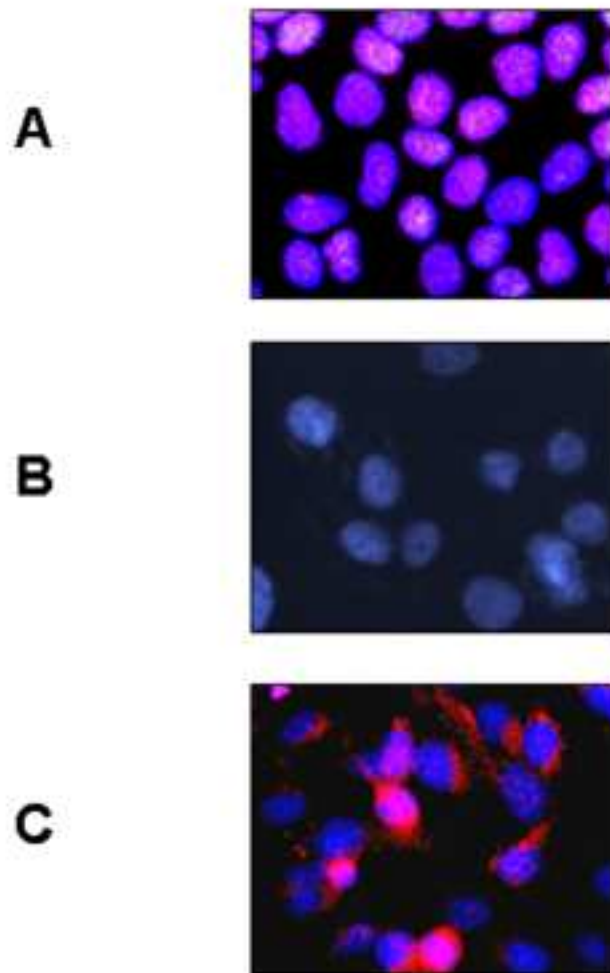


Figure 2

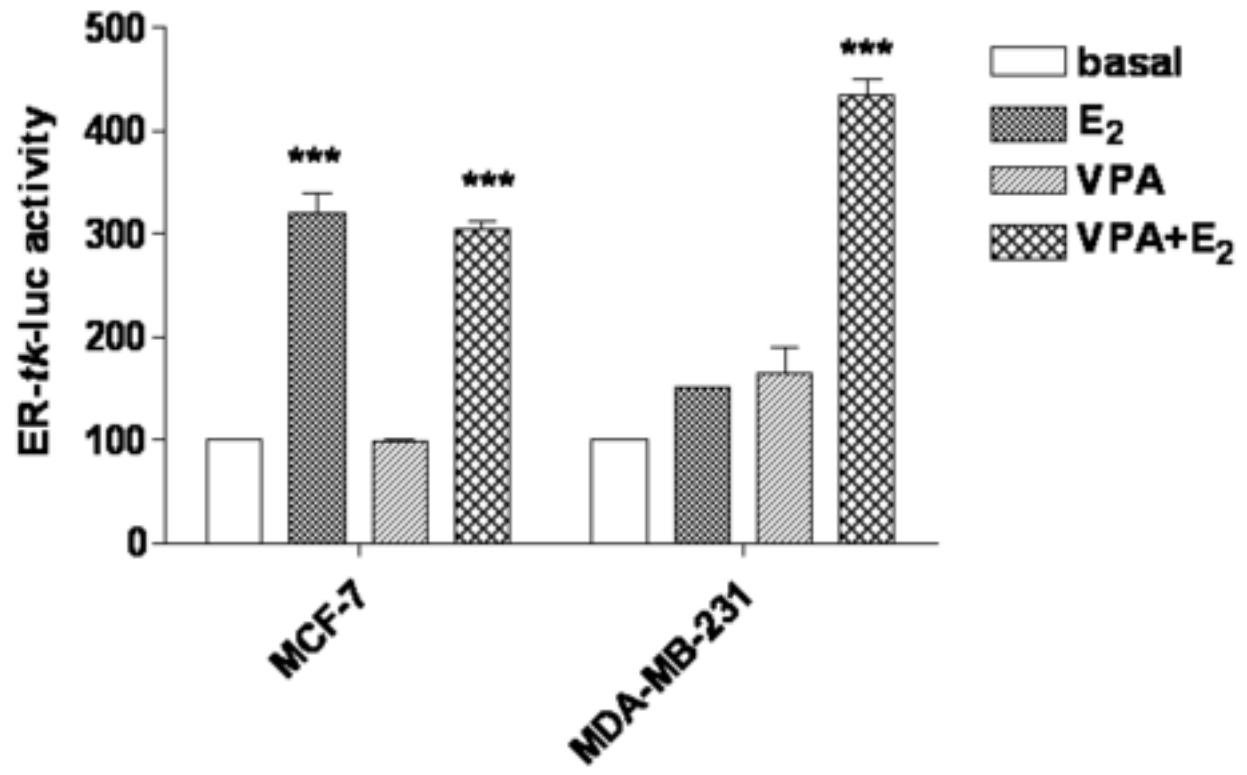


Figure 3

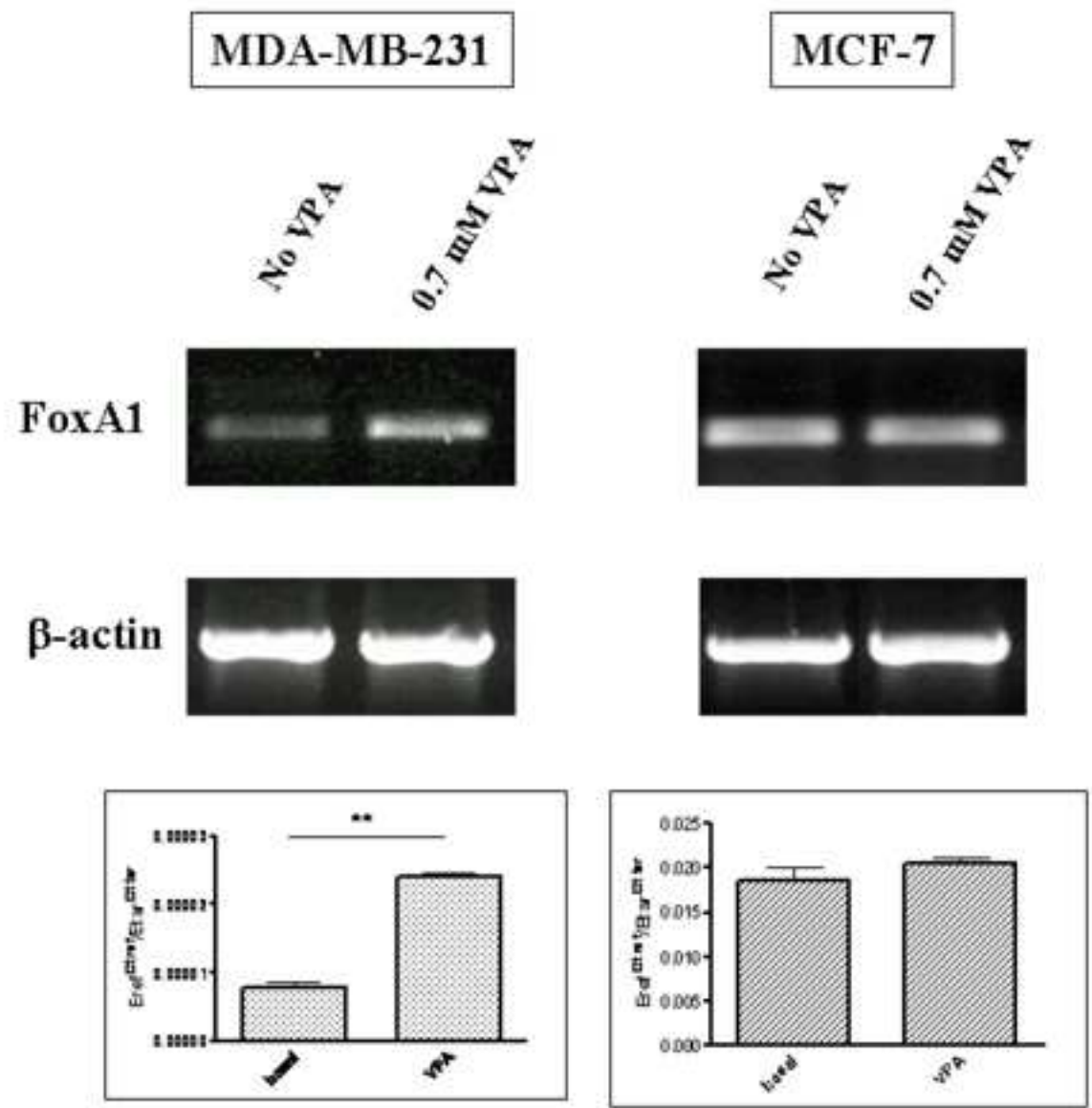


Figure 4

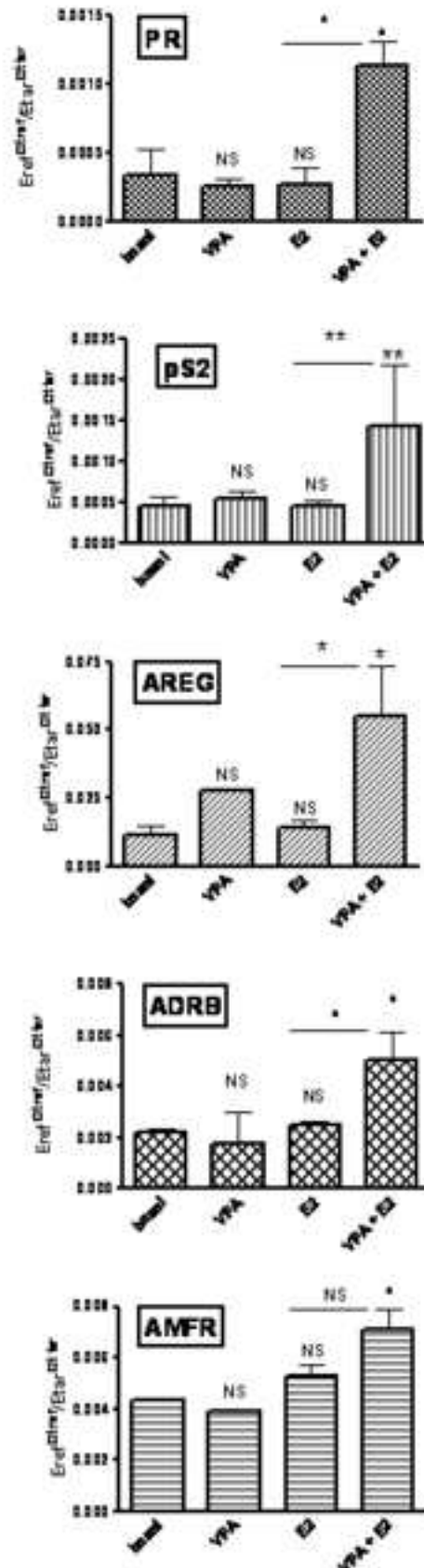


Figure 5

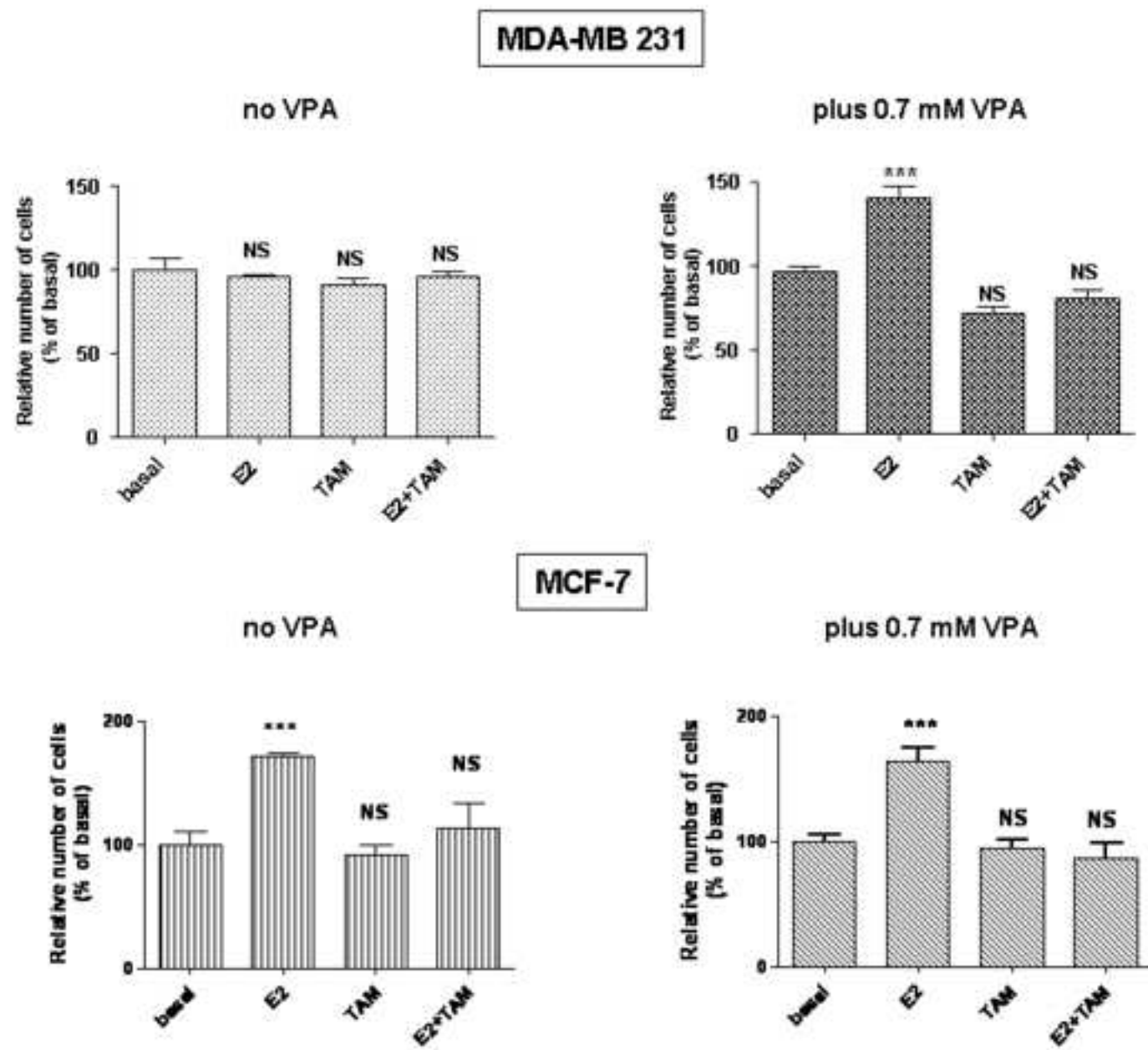


Figure 6