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Preclinical study

Effect of Vinca alkaloids on ERα levels and Estradiol-induced responses in MCF-7 cells

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Summary

Vinca alkaloids (VAs) such as Vincristine, Vinblastine and Vinorelbine are antineoplastic drugs that inhibit tubulin polymerisation into microtubules, induce mitotic G_2/M arrest, activate c-Jun N-terminal kinase (JNK) and induce apoptosis. Although there are many studies evaluating the effect of VAs on breast cancer patients, until now little was known about how these compounds and estradiol signaling pathways might interfere. In this report, we show for the first time that VAs decreased ERα protein levels in the human breast cancer cell line MCF-7; VAs induced a parallel decrease in estrogen receptor mRNA. All the VAs tested inhibited estradiol (E_2) mediated transactivation at ERE-driven promoters. E_2 inhibited VAs-induced AP1 stimulation in MCF-7, but this inhibition was not observed when E_2 is added 24 h in advance of VAs treatment. In contrast to the reported preventing effect over taxol-mediated apoptosis, E_2 did not prevent VAs-induced cell death and interestingly, addition of E_2 24 hours in advance of VAs treatment resulted in an increase of the number of cells undergoing apoptosis. Similar results were observed when E_2 is replaced by other proliferation signals such as EGF. These results demonstrate that in the breast cancer cell-line MCF-7, E_2-induced proliferation before VAs treatment enhances the apoptotical response to VAs which might have important implications in clinica.

Abbreviations: AP1: activator protein 1; CaM: calmodulin; CB: cytochalasin B; E_2: estradiol; EGF: epidermic growth factor; ERE: estrogen response element; GAPDH: glyceraldehydes-3-phosphate dehydrogenase; JNK: c-jun N-terminal kinase; Luc: luciferase; MIAs: microtubule interfering agents; Nb: navelbine (vinorelbine); Noc: nocodazole; PLB: passive lysis buffer; TMRM: tetra methyl rhodamine methyl ester; VAs: vinca alkaloids; Vb: vinblastine; Vc: vincristine; W7: N-(6-aminohexyl)-5-chloro-naphtalene sulfonilamide hydrochloride

Introduction

Microtubule interfering agents (MIAs) are widely used in cancer chemotherapy. *Vinca* alkaloids such as Vinblastine (Vb) and Vincristine (Vc) or the semisynthetic derivative Vinorelbine (Navelbine, Nb) alter microtubule dynamics by binding to beta-tubulin monomers and dimers resulting in G2-M arrest, inhibition of cell proliferation, and apoptosis [1–3]. Vinblastine introduces a wedge at the interface of two tubulin molecules and thus interferes with tubulin assembly [4]. Several studies have reported that *Vinca* alkaloids cause significant activation of JNK prior to caspase3 activation [5]. Vb also causes selective effects on AP1 phosphorylation and composition [6] and it has been described that the c-Jun N-terminal kinase/AP-1 pathway is required for efficient apoptosis induced by this agent [7]. Vc treatment of the breast cancer cell-line HBL100 leads to transcriptional repression of BRCA1 and induction of JNK1 and c-jun [8].

In the breast cancer cell line MCF-7, E_2 prevents UV- or taxol-induced apoptosis [9]. These agents stimulate the phosphorylation of Bcl-2 and Bcl-X_L [9,10]. Vb also induces JNK-mediated phosphorylation and consequently inactivation of Bcl-2 and Bcl-X_L in KB-3 cells [11]. Active Bcl-2 and Bcl-X_L have been proposed to prevent cytochrome C release from mitochondria [12], and therefore, activation of caspases [9]. Considering that it has been reported that E_2 can stimulate both transcription and protein synthesis of Bcl-2 [13], it is likely that E_2 can

prevent UV- and taxol-induced apoptosis by regulating both the activity and levels of this protein.

We have previously reported that calmodulin (CaM) is a selective modulator of estrogen receptors. As a consequence, E_2-mediated transactivation was inhibited in MCF-7 by the calmodulin antagonist W7 [14]. It is known that *Vinca* alkaloids interact with CaM [15], and some enzymes which activity is modulated by CaM are affected by VAs [16,17]. It has been suggested that some negative side effects of VAs observed in clinica may be due to interaction of this compounds with CaM [18]. Clinical reports associate the response to chemotherapy in operable breast cancer with no expression of estrogen receptors [19] and it has also been described that navelbine induces cellular differentiation and influences the estrogen receptor status of Lewis lung carcinoma [20].

In this work we demonstrate an effect of VAs on ERα protein and mRNA levels and also on ERα transcriptional activity in the human breast cancer cell-line MCF-7. We also show that E_2 inhibits Vb- and Vc-induced AP1 activation in MCF-7 cells when cells are co-treated with E_2 and VAs. Moreover, E_2 does not prevent VAs-induced apoptosis, as it has been reported for UV- and taxol-induced apoptosis and, interestingly, E_2 pretreatment for 24 h prior to VAs addition results in a higher ratio of cell death.

Materials and Methods

Materials

17β-Estradiol, vinblastine, vincristine, EGF, taxol, nocodazole, cytochalasin B and other reactives were purchased from Sigma. Navelbine was purchased from GlaxoSmithKline. MMLV Reverse Transcriptase was purchased from Invitrogen. Anti-ERα was purchased from Babco.

Plasmids

The plasmid 3X-ERE-TATA-Luc was kindly provided by Dr. S. Safe from the Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, Texas. *Renilla* luciferase plasmid (pRL)-TK (Promega Corp., Madison, WI), and Δcoll 73-Luc [21] were also used in this work.

Immunoblotting

Equal amounts of protein lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane and blocked in a buffer containing 5% non-fat milk. Immunoblots were probed with anti-estrogen receptor (ER7 Babco 1/500). Complexes were visualized with an anti-mouse horseradish peroxidase-conjugated secondary antibody and bands were revealed using ECL (Amersham) according to manufactures instructions.

RNA extraction and cDNA synthesis

MCF-7 cells were treated with either vehicle, Vc, Vb or Taxol for 24 h. Total RNA was extracted using Tri-Reagent (Sigma) and the yield was measured spectrophotometrically. Quality and integrity of total RNA was assessed on 2% agarose gels. Single stranded cDNA was synthesized from 2 μg of total RNA using MMLV Reverse Transcriptase. All the procedures were performed following the manufactureŕs protocol.

Real time RT-PCR

Real time PCR was done using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Cheshire, UK). 10 μl of the reaction mixtures contained: 1×SYBR® Green PCR Master Mix, 1% RT product, 300 nM of each primer and nuclease free water. No template controls were also prepared. PCR conditions were an initial denaturation and DNApol activation step of 95 °C for 10 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing-extension for 1 min at 60 °C. All samples were amplified in triplicate and real-time PCR experiments were performed from samples of three independent experiments. ERα mRNA levels were normalized to GAPDH as an internal control. The following primers were designed with the Primer Espress® v2.0: ERα Forward 5′-CCGCTCATGATCAAACGCTCTAA-3′ and Reverse 5′-ATGGAGGGTCAAATCCACAAAG-3′. GAPDH Forward 5′-TCTGGTAAAGTGGATATTGTTG-3′ and Reverse 5′-GATGGTGATGGGATTTCC-3′.

Cell culture and transient transfection assays

MCF-7 cells were propagated in RPMI 1640 medium containing 25 mM HEPES/NaOH (pH 7.3), 100 μg/ml gentamicin and 10% heat-inactivated fetal bovine serum (FBS) (Roche Molecular Biochemicals) in a humidified atmosphere of 5% CO_2 at 37 °C. Before transfection, MCF-7 cells were seeded in twelve-well plates and incubated 12–18 h at 37 °C. Then, cells were transferred to phenol-red free RPMI 1640 containing 0.5% charcoal/dextran-treated FBS (sFBS) and maintained for 2 d. At 60–80% confluency, cells were transfected with 0.5 μg of ERE-driven or AP1-driven reporter plasmids and 50 ng of an internal control *Renilla* luciferase plasmid, pRL-TK (Promega, Madison, WI) using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals), following the manufacturer's instructions. After 18–24 h, medium was renewed and cells were stimulated during 24 h with different chemicals, as indicated.

HeLa cells were propagated in phenol-red DMEM (Life Technologies) containing 100 μg/ml gentamicin and 10% heat-inactivated fetal bovine serum (FBS) (Boehringer Roche Molecular Biochemicals) in a humidified atmosphere of 5% CO_2 at 37 °C. Before transfection, HeLa cells were seeded in twelve-well

plates and incubated 12–18 h at 37 °C. Then, cells were transferred to phenol-red free Dulbecco's Modified Eagle's Medium containing 0.5% (sFBS) and maintained for three days. At 60–80% confluency, cells were transfected with 0.75 μg of ERE-driven or AP1-driven reporter plasmids, 0.1 μg of ER expression vector and 75 ng of an internal control *Renilla* luciferase plasmid (pRL-TK), using FuGENE 6 Transfection Reagent from Roche Molecular Biochemicals following the manufacturer's protocols. After 18–24 h, medium was renewed and cells were stimulated during 24 h with different chemicals, as indicated.

For luciferase assays, cells were washed with PBS and lysed by the addition of 200 μl of PLB 1X as recommended by Dual Luciferase System (Promega). After centrifugation, supernatants were saved as extracts. Luciferase activities were determined in 20 μl of each cellular extract and experimental values were normalized to *Renilla* luciferase activity, in order to correct for differences in transfection efficiency.

The results represent the means ± SD of three independent experiments performed at least in duplicate.

Mitocondrial membrane potential

To measure apoptosis, MCF-7 cells were seeded in six-well plates and incubated 12–18 h at 37 °C. Then, cells were cultured in phenol-red free RPMI 1640 containing 0.5% charcoal/dextran-treated FBS (sFBS) and maintained for 2 days. When necessary, E_2 was added 24 h prior to treatment with VAs. Cells were maintained for 24 additional hours and assessed for mitochondrial membrane potential with tetramethyl rhodamine methyl ester (TMRM; Molecular Probes), a fluorescent dye rapidly responsive to mitochondrial membrane potential as described [22]. The difference in staining for cells with polarized and depolarised mitochondria typically is at least one order of magnitude.

Statistical analysis

The data on ERα expression, luciferase activity and cell proliferation are expressed as the mean ± standard errors of the mean (SEM). Statistical differences between groups were processed by one way analysis of variance (ANOVA) followed by the Newman–Keuls test. Results were considered as statistically significant at $p < 0.05$

RESULTS

Effect of VAs treatment on the level of ERα protein on MCF-7 cells

To determine whether VAs alter the expression of ERα, MCF-7 cells were treated with 1 μM of either Vb or Vc for 24 h, and the level of ERα protein was determined by Western blot analysis. ERα protein levels declined about 60% after treatment with these compounds (Figure 1a, lanes 2 and 3). MCF-7 cells treatment with other MIAs (taxol, nocodazole) or cytochalasin B did not result in a decrease of the receptor protein levels (Figure 1a, lanes 4, 5 and 6). In a complementary experiment, MCF-7 cells were treated with 1 μM of Vc, and the levels of ERα protein were determined 3, 6, 12 and 24 h after Vc treatment. As shown in Figure 1b ERα protein levels declined after 12 h of treatment with the *vinca* alkaloid. Membranes were stripped and blotted with antibodies directed against *β*-actin as internal control, to demonstrate that identical amounts of total protein were resolved in each lane (data not shown).

Effect of VAs treatment on the steady-state level of ERα mRNA on MCF-7 cells

The steady-state levels of ERα mRNA isolated from MCF-7 cells treated with vehicle, 1 μM of either Vb or Vc, or 1 μM of taxol for 9 h were determined by real time PCR; as reference, GAPDH transcripts were also quantified. The results shown in Figure 2 prove that treatment with 1 μM of either Vb or Vc resulted in a 72% decrease in the ERα mRNA levels which correlates with the magnitude of the effect of VAs on ERα protein levels (Figure 2, lanes 2 and 3), whereas treatment with 1 μM of taxol results in an increase of the steady-state levels of ERα mRNA (Figure 2, lane 4). These results are in agreement with previous reports [23].

Vinca alkaloids inhibit the reporter gene activity induced by E_2 from a consensus ERE in MCF-7

MCF-7 cells were transiently transfected with 3X-ERE-TATA-Luc reporter plasmid and stimulated with 10 nM E_2. Estradiol induced a significant increase (5-fold) in ERα mediated transactivation (Figure 3, bar 5). Treatment with 1 μM of Nb, Vb or Vc alone did not activate ERα-mediated transcription (Figure 3, bars 2, 3 and 4).

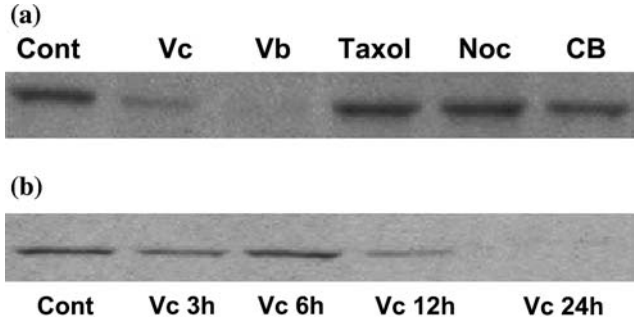

Figure 1. Effect of VAs on the steady-state level of ERα protein. (a) Synchronized MCF-7 cells in RPMI 1640 medium containing 0.5% charcoal, dextran-treated fetal calf serum were stimulated for 24 h with vehicle (control), 1 μM of Vc, 1 μM of Vb, 1 μM of Taxol, 1 μM of Nocodazole or 1 μM of Cytochalasin B. (b) ERα protein levels were determined 3, 6, 12 and 24 h after Vc treatment. The relative amount of endogenous ERα present in whole cell extracts was analyzed by immunoblotting with antibodies directed against ERα.

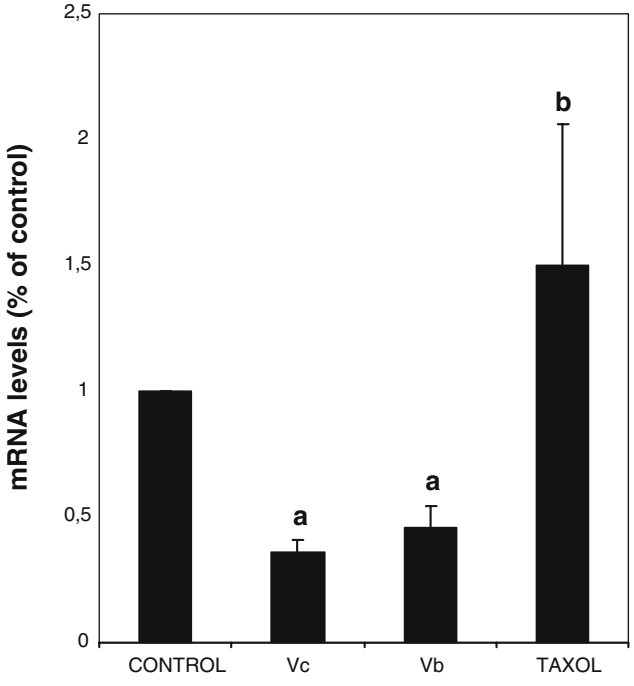

Figure 2. Effect of VAs on the steady-state level of ERα mRNA Synchronized MCF-7 cells in RPMI 1640 medium containing 0.5% charcoal, dextran-treated fetal calf serum were stimulated for 24 h with either vehicle (control), 1 μM of Vb, 1 μM of Vc, or 1 μM of taxol. Total mRNA was isolated and single stranded cDNA was synthesized from 2 μg of total RNA followed by real time PCR. a, $p < 0.01$ versus control; b, $p < 0.05$ versus control.

When MCF-7 cells were treated with both 10 nM of E_2 plus either 1 μM of Nb, Vb or Vc we observed a significant decrease in luciferase activity (Figure 3, compare bars 6, 7 and 8 to lane 5). In this cell-line, VAs significantly inhibited the transcriptional activation induced by E_2 (58, 72 and 69% respectively). These results are similar to those obtained by using W7 [14], and are in agreement with the hypothesis that VAs might act as calmodulin antagonists.

E_2 co-treatment, but not pre-treatment inhibits AP1 activation induced by Vinca alkaloids in MCF-7

It has been extensively reported that VAs treatment results in activation of JNK, therefore, we decided to test whether or not E_2 treatment might alter the AP1 response to VAs. We examined the ability of E_2 to influence VAs transcriptional activity mediated at AP1 sites by transiently transfecting MCF-7 cells with Δcoll 73-Luc reporter plasmid. In agreement with previous reports [24], Vb and Vc enhanced AP1 transcriptional activity (3.3-fold) in MCF-7 cells (Figure 4A and B, lane 2). Interestingly, both Vb- and Vc-mediated AP1 activation was inhibited (61 and 68%) when cells were co-treated with E_2 (Figure 4A and B, lane 3). These results are in agreement with an E_2-induced reduction of 50–67% in the stimulated JNK activity observed in response to UV or taxol [9].

Interestingly, when cells are pre-treated for 24 h with 100 nM of E_2, addition of either 1 μM of Vb or Vc stimulated AP1-mediated transactivation to the same levels as treatment with VAs alone (Figure 4A and B, compare lanes 2 and 4). In other words, the E_2 inhibitory effect over Vb- and Vc-induced AP1 is abolished when E_2 treatment preceeds VAs addition. Co-treatment with EGF (another agent that stimulates AP1 pathways)

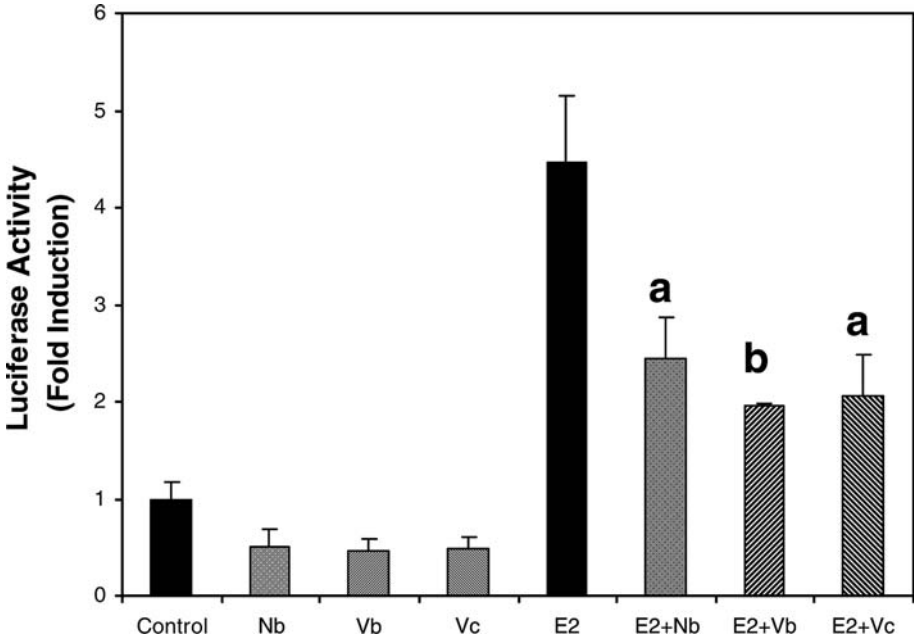

Figure 3. *Vinca* alkaloids inhibit the E_2-dependent transcriptional activation in the human breast cancer cell-line MCF-7. MCF-7 cells were transiently transfected with 0.5 μg of reporter plasmid 3X-ERE-TATA-Luc and 50 ng of control reporter vector pRL-TK. 24 h after transfection, cells were further treated for 24 h with vehicle, 10 nM Estradiol (E_2), 1 μM of either navelbine (Nb), vinblastine (Vb) or vincristine (Vc) alone or 1 μM E_2 plus 1 μM navelbine (E_2 + Nb), vinblastine (E_2 + Vb) or vincristine (E_2 + Vc) as indicated. The data are reported as fold-induction with respect to untreated cells, which were arbitrarily assigned as 1. The bars represent the means ± SD of three independent experiments run in duplicates. a, $p < 0.01$ versus control; b, $p < 0.001$ versus control.

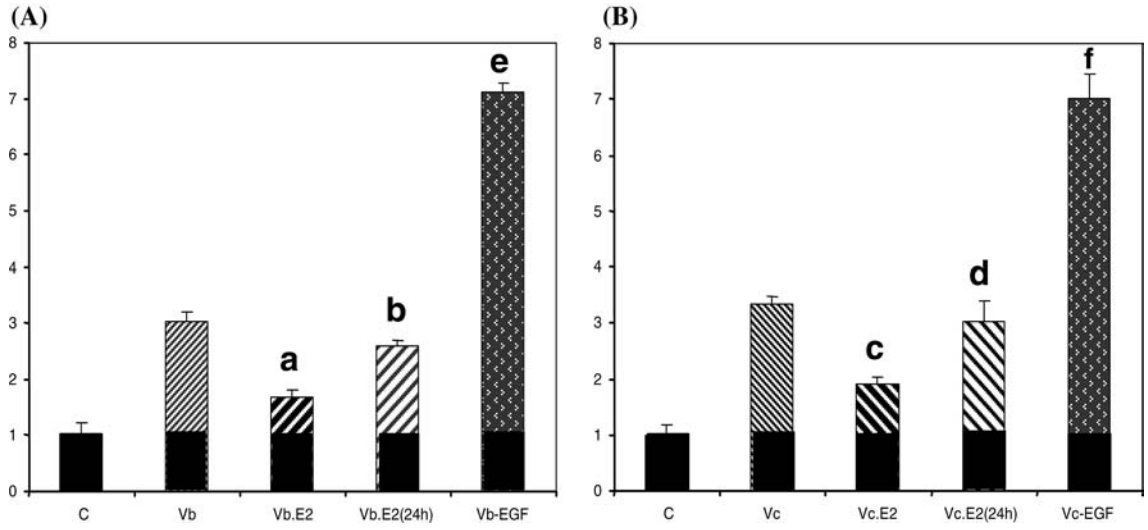

Figure 4. Effect of E_2 on Vinblastine- (A) or Vincristine-dependent activation (B) at an AP1 element in MCF-7 cells. MCF-7 cells were transfected with 50 ng of internal control plasmid pRL-TK and 0.5 μg of AP1-containing reporter plasmid (Δcoll. 73-Luc). a, Cultures were stimulated for 24 h with vehicle (Control), 1 μM vinblastine (Vb), 1 μM vinblastine plus 100 nM Estradiol (Vb + E_2), 24 h pre-treatment with 100 nM Estradiol before addition of 1 μM vinblastine ($E_{2(24\ h)}$ + Vb) or 1 μM vinblastine + 10 μg/ml EGF (Vb-EGF) as indicated. b, Same as in a, but vincristine instead of vinblastine. Luciferase activity was determined and the data are reported as fold-induction with respect to untreated cells, which were arbitrarily assigned as 1. The bars represent the means ± SD of three independent experiments run in duplicates. a, $p < 0.01$ versus Vb; b, $p < 0.01$ versus (Vb + E_2); c, $p < 0.01$ versus Vc; d, $p < 0.01$ versus (Vc + E_2); e, $p < 0.001$ versus Vb; f, $p < 0.001$ vs Vc.

and either Vb or Vc resulted in higher levels of AP1 transactivation (Figure 4A and B, lane 5).

E_2 inhibits VAs dependent activation at an AP1 element through ERα

We next studied whether the inhibitory effect of E_2 on VAs dependent activation at AP1 sites was dependent of ERα expression by transiently transfecting HeLa cells with Δcoll 73-Luc reporter plasmid. We found that AP1 activity was increased by 1 μM of Vc in an ERα-independent fashion (Figure 5, left panel). However, E_2 significantly inhibited the activity of AP1 in ERα-transfected cells (Figure 5, right panel). These results indicate that E_2 counteract the stimulatory effect of VAs on AP1 only in cells expressing ER (compare Figure 5, right and left panels).

Pre-treatment with E_2 enhances apoptosis in MCF-7 cells

It has been previously reported that E_2 prevents UV- and taxol-mediated cell death [9]. We decided to address

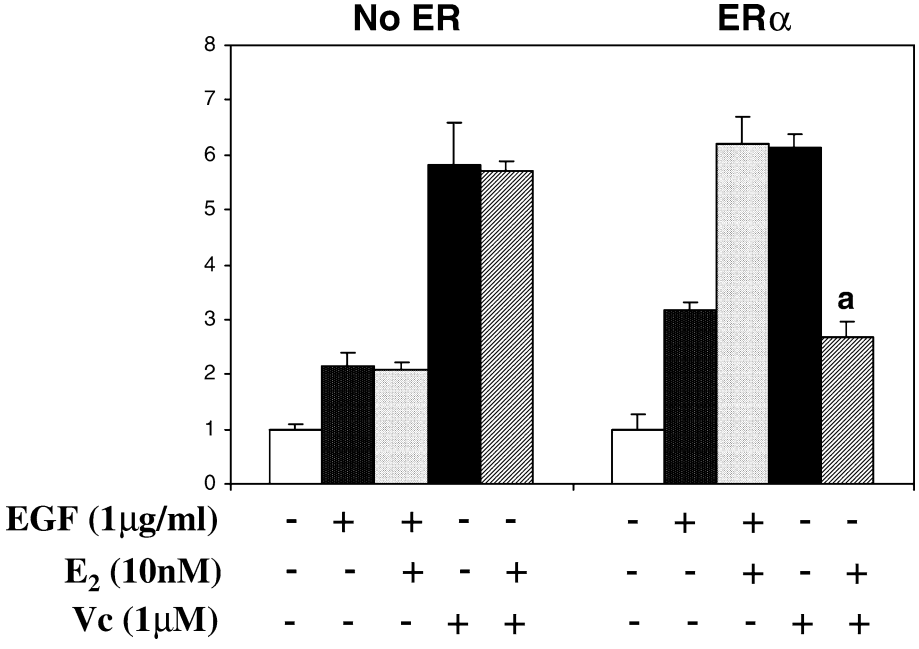

Figure 5. E_2 inhibits VAs dependent activation at an AP1 element through ERα. HeLa cells were transfected with 0.1 μg of the ERα expression vector, 50 ng of internal control plasmid pRL-TK and 0.75 μg of the AP1-containing reporter plasmid (Δcoll. 73-Luc). Cultures were stimulated for 48 h with 1 μg/ml EGF, 10 nM E_2 and 1 μM Vc as indicated. The data are reported as fold-induction relative to untreated cells, which were arbitrarily assigned as 1. The bars represent the means ± SD of three independent experiments run in duplicates. a, $p < 0.01$ versus Vc.

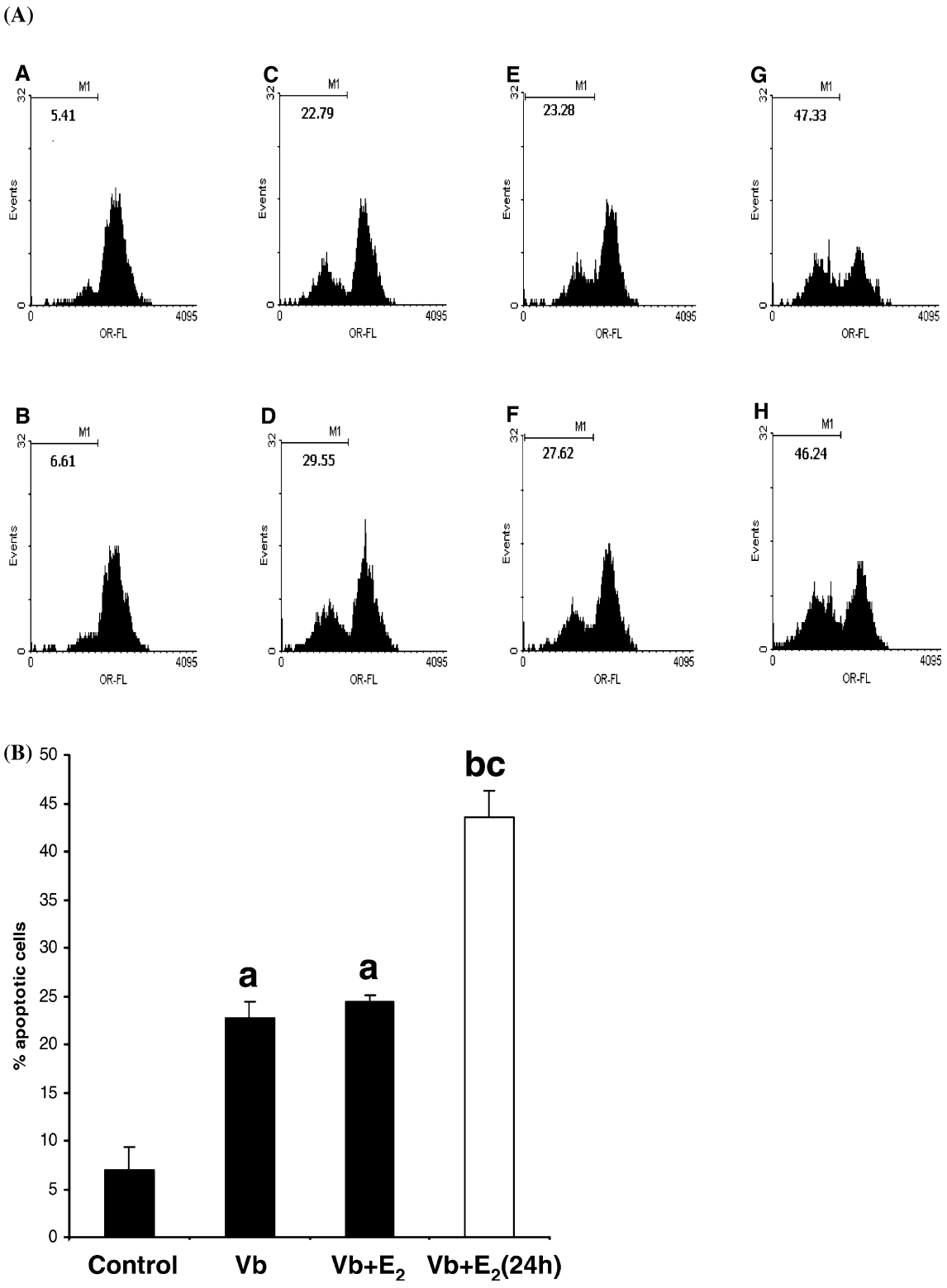

Figure 6. Pre-treatment with E_2 potentiates *Vinca* alkaloids-induced apoptosis in MCF-7 cells. (a) MCF-7 cells were seeded in twelve-well plates and incubated 12–18 h at 37°C. Then, cells were transferred to phenol-red free RPMI containing 0.5% charcoal/dextran-treated FBS (sFBS) and maintained for 48 h. Cells were treated with vehicle (A), 100 nM E_2 (B), 1 μg/ml vinblastine (C) 1 μM vincristine (D), 1 μg/ml Vb + 100 nM Estradiol (E), 1 μM vincristine + 100 nM Estradiol (F). In G and H, cells, were pre-treated with 100 nM Estradiol and 24 h later 1 μM vinblastine or vincristine was added. Loss of mitochondrial potential was determined by flow cytometry as described under Materials and Methods. The cells with low mitochondrial potential are those located under the bar marked as M1. (b) The percentage of apoptosis under the different treatments is represented. a, $p < 0.01$ versus control; b, $p < 0.01$ versus Vb ; c, $p < 0.01$ versus (Vb + E_2).

the effect of E_2 treatment, and also, whether or not the timing of E_2 treatment might alter the number of cells undergoing apoptosis. The results obtained are shown in Figure 6. As expected, treatment with both 1 μM of either Vb or Vc significantly increased the number of cells that entered into apoptosis (Figure 6, panels C and

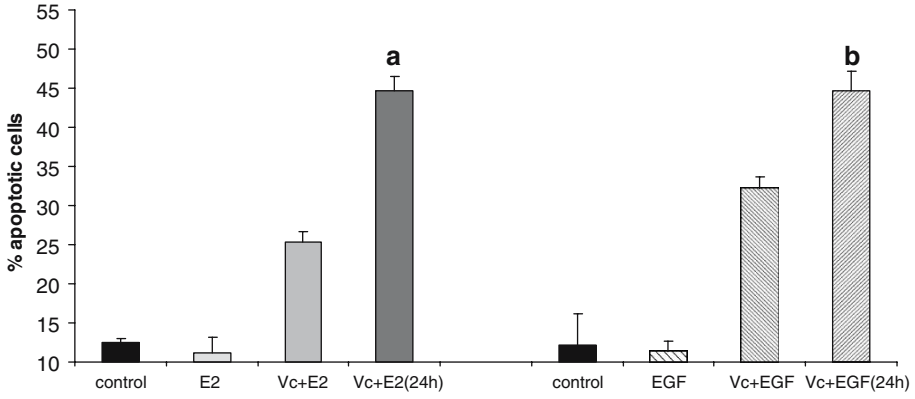

Figure 7. Pre-treatment with EGF also potentiates *Vinca* alkaloids induced apoptosis in MCF-7 cells. MCF-7 cells were seeded in twelve-well plates and incubated 12–18 h at 37 °C. Then, cells were transferred to phenol-red free RPMI containing 0.5% charcoal/dextran-treated FCS (sFCS) and maintained for 48 h. Cells were treated with vehicle (Control), 100 nM E_2 (E2), 1 μM vincristine plus 100 nM E_2 (Vc + E2), 1 μg/ml vincristine after 24-h pre-treatment with 100 nM E_2 (Vc-$E2_{(24\ h)}$), 1 μg/ml EGF (EGF), 1 μM vincristine plus 1 μg/ml EGF (Vc-EGF) and 1 μM vincristine after 24 h pre-treatment with 1 μg/ml EGF (Vc-$EGF_{(24\ h)}$). Loss of mitochondrial potential was determined by flow cytometry as described under Materials and Methods. a, $p < 0.01$ versus (Vc + $E_{2)}$; b, $p < 0.01$ versus (Vc + EGF).

D), when compared to cells treated with either vehicle or E_2 (Figure 6, panels A and B); Interestingly, E_2 does not prevent VAs-induced apoptosis (Figure 6, panels E and F) and, most importantly, when cells are pre-treated for 24 h with E_2 (Figure 6, panels G and H), the number of cells that entered into apoptosis is significantly higher than that observed when cells were treated only with VAs, or co-treated with E_2 + VAs. The percentage of apoptotic cells is represented in Figure 6b.

EGF pre-treatment 24-h prior to VAs addition also results in increased levels of apoptosis in MCF-7 cells

We next addressed if EGF pre-treatment might have a similar effect to that of E_2 in VAs-induced apoptosis in MCF-7. Results are shown in Figure 7. EGF alone does not induce apoptosis (Figure 7, lane 6). Similarly to E_2, EGF does not prevent VAs-induced apoptosis (Figure 7, lane 7) and, when cells are pre-treated for 24 h with EGF plus Vc (Figure 7 lane 8) the number of cells that entered into apoptosis is much higher. Identical results have been also obtained when the experiments were performed using Vb instead of Vc (not shown).

Discussion

Vinblastine (Vb) and vincristine (Vc) are alkaloids found in *Catharantus roseus*, formerly classified as *Vinca rosea*, which led to these compounds becoming called V*inca* alkaloids [25]. They, as well as the semisynthetic derivatives of vinblastine, vindesine and vinorelbine [26,27], all work by inhibiting mitosis in metaphase. These alkaloids are microtubule-interfering agents (MIAs) that bind to beta-tubulin, thus preventing the cell from making the mitotic spindle necessary to move the chromosomes around as the cell divides [28]. *Vinca* alkaloids have been widely used in cancer chemotherapy with promising results. It has been well established that the main action of these agents is to arrest the cell cycle at the G_2/M phase. It has also been reported that Vb and Vc activate the c-Jun N-terminal kinase (JNK/SAPK) signalling pathway in a variety of human cells. MIAs also activate both Ras and apoptosis signal-regulating kinase (ASK1) [29]. Another report has showed that Vb-induced phosphorylation of Bcl-2 and Bcl-X_L is mediated by JNK, demonstrating a direct role of the JNK pathway in apoptotic regulation through Bcl2/Bcl-X_L phosphorylation [11]. In KB-3 cells the activation of JNK by Vb and Vc is prior to caspase 3 activation [5]. VAs can also cause highly selective effects on AP1, for example, c-Jun and Fra1 expression is greatly increased and these proteins become phosphorylated, while Jun B remains undetectable [6].

Many reports in the literature refer to clinical trials in which patients are treated with these compounds. It has been reported that low-dose chemotherapy (including Vc) in addition to tamoxifen does not improve the prognosis of stage II breast cancer patients with hormone-responsive tumors [30]. A clinical study investigated the efficacy of a combination chemotherapy with vinorelbine, 5-fluorouracil and folinic acid in operable breast cancer, concluding that positive responses were significantly associated with no expression of estrogen and/or progesterone receptors [19]. Another study showed that negative ER detection by immunohistochemistry was highly correlated with chemosensitivity [31]. In summary, *vinca* alkaloids inhibit mitosis, are used as chemotherapy agents in many types of tumors including breast cancer, stimulate JNK activation, produce changes in AP1 composition, and promote apoptosis. Also when used in operable breast cancer, the patients that are ER positive do not respond well to the treatment. Moreover, VAs in addition to tamoxifen do not improve the prognosis of patients with hormone-responsive tumors.

There are several reasons to link VAs and estrogen receptors: we have recently showed that only ERα interacts with calmodulin whereas ERβ does not; as a consequence, ERα but not ERβ can be specifically inactivated by calmodulin, such as W7. *Vinca* alkaloids have been also reported to interact with calmodulin in a way that alters the activity of calmodulin-interacting enzymes such as phospho-fructo kinase [16].

Since several CaM antagonists have been reported to promote ER degradation, the first purpose of this study was to determine the effect of VAs on ERα expression. We have examined the VAs effects on ERα protein and mRNA levels. Our results prove that VAs decreased the steady-state levels of ERα protein and mRNA.

Next, we decided to investigate the effect of Nb, Vb and Vc over ERE-driven promoters in the breast cancer cell-line MCF-7. We have recently reported that the CaM antagonist W7 inhibits E_2-mediated transcription in MCF-7 cells. VAs treatment of MCF-7 cells results in inhibition of E_2-mediated transcription, indicating that VAs might also act as calmodulin antagonists over ERα-mediated transcription.

We decided to study further how VAs and E_2 signalling pathways interfere. In agreement with previous reports [25], VAs induced AP1 activation (3.3-fold) in MCF-7 cells at similar levels of those previously reported for taxol [9]. E_2 partially reverses this induction when cells are co-treated with either Vb or Vc plus E_2 and this result is similar to that obtained for UV- and taxol-induced JNK activation [9]. Importantly, E_2 inhibition of Vb- and Vc-induced AP1 transactivation is abolished if cells are pre-treated for 24 h with the hormone before adding the VA. In transient transfection experiments performed in HeLa cells, we found that E_2 counteract the stimulatory effect of VAs on AP1 only in cells expressing ERα.

It has been previously reported that UV- and taxol-induced apoptosis in MCF-7 cells is significantly reversed by incubation with E_2, consequently with the idea that E_2 is a survival factor for ER(+) breast cancer cells. This effect is rapid and seems to be mediated through the plasma membrane estrogen receptor. E_2 partially, but significantly, prevents UV- or taxol-induced JNK activation [9]. We have decided to address if E_2 could have similar effects over VAs-induced apoptosis. Interestingly, in our hands, E_2 is not protecting the cells from VA-induced apoptosis, which differs from the results reported by Razandi et al. [9]. The apparent discrepancy between our results and those reported by these authors might have several explanations: (i) the apoptosis-inducing agent used in both cases is different; (ii) opposite effects have already been described for VAs and paclitaxel; for example, it has been reported that ERK2 is activated by vinblastine and inhibited with paclitaxel [32]; (iii) we found that ERα protein and mRNA levels are diminished by VAs but not by taxol. Also, the difference in the results might also be explained since we measured apoptosis after incubation for longer times and we performed our experiments in serum-depleted medium.

Another important observation is that 24 h pre-treatment with E_2 significantly increased the number of cells that entered into apoptosis; This result might be explained since we performed our experiments in serum-depleted medium where cells are synchronised, therefore they will proliferate when E_2 is added and likely, more cells will die when the signal that trigger apoptosis (Vb or Vc) is preceded by a signal of proliferation (E_2). Accordingly to this, we have obtained similar results when E_2 is replaced by EGF. Addition of EGF 24 h prior to Vb or Vc treatment results in increased levels of apoptosis. The lack of correlation between the effect of E_2 on VAs-induced AP1 activation, and VAs-induced apoptosis that we report here might be explained since recently, it has been reported that MIAs promote phosphorylation of Bcl-2, a prominent anti-apoptotic marker, but this phosphorylation is unaffected by JNK inhibition, providing evidence for a novel signalling pathway connecting microtubule damage to apoptosis induction [33].

In summary, we have demonstrated an inhibitory effect of VAs on ERα levels and activity in human breast cancer cells. We also proved that E_2 inhibits Vb- and Vc-induced AP1 activation in MCF-7 cells only when cells are treated at the same time with E_2 and VAs. The effect of E_2 does not take place when E_2 is added 24 h prior to VAs treatment. In our hands, E_2 does not prevent VAs-induced apoptosis, as it has been reported for UV- and taxol-induced apoptosis and, interestingly, E_2 pre-treatment for 24 h prior to VAs addition results in a higher ratio of cell death. The effect of prior estradiol exposure increasing responses to cytotoxic drugs has been described in clinic [34] and in cell culture models [35,36].

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