Antioxidants and doxorubicin supplementation to modulate CD14 expression and oxidative stress induced by vitamin D₃ and seocalcitol in HL60 cells

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Abstract. 1α,25-dihydroxyvitamin D₃ (VD₃) and the EB1089 analog are well known for their roles in the modulation of proliferation and the differentiation of several malignant cells. In addition, VD₃ or EB1089 displayed a high disposal of oxidant features and the ability to cause release of reactive oxygen species (ROS). We attempted to enhance HL60 cell differentiation and to limit ROS generation, by the association of deltanoids with doxorubicin and the antioxidants catalase (CAT), superoxide dismutase (SOD) and N-acetyl cystein (NAC). Differentiation of HL60 cells into monocytic lineage was studied by expression of mRNA, protein CD14 and functional differentiation by the nitroblue tetrazolium assay. The 2',7'-dichlorodihydrofluorescein diacetate (H₂-DCFDA) dye allowed to evaluate in situ ROS generation. When associated with 0.1 nM EB1089, 15 nM doxorubicin induced an increase of differentiated cell percentage from 29% to 87% and did not affect VD₃-treated cells. The association with doxorubicin also induced a significant increase of ROS release (p<0.05) versus VD₃ and EB1089-treated cells. These results correspond to additivity of individual effects of doxorubicin and deltanoids. Antioxidant agents (10 nM NAC, 50 U/ml SOD or 2000 U/ml CAT) were associated with 10 nM VD₃ or 1 nM EB1089 for 72 h. Compared to VD₃ and EB1089 treatments, associations with antioxidants induced a slight increase of differentiated cells and a significant increase of CD14 mRNA. The highest differentiation effect occurred in the case of the EB1089-NAC association. Antioxidants induced a decrease (p<0.05) in ROS release generated by VD₃ or EB1089 near the level of untreated cells. Thus, antioxidant agents demonstrated a protective effect against VD₃ and EB1089 oxidative cytotoxicity and an enhancement of the monocyte differentiation. Combinations of antioxidants with deltanoids could dissociate the oxidative stress and differentiation.

Introduction

1α,25-dihydroxyvitamin D₃ (VD₃) is well known for its regulatory role in the modulation of proliferation and differentiation in several normal and malignant cell types (1). In vivo and in vitro anticancer activities of VD₃ have been commonly attributed to cytostatic and cytotoxic effects on cancer cells. VD₃ induces apoptosis in breast cancer cells and decreases invasiveness of a number of cell lines in vivo. In vitro studies demonstrated that VD₃ inhibits cell growth and promotes cell differentiation in tumor cell types such as breast, colon, prostate, glioma, pancreas and neuroblastoma (2,3). Differentiation of immature myeloid cells into granulocytic-monocytic lineages resulted in the appearance of specific markers in presence of different agents (4). VD₃ induces HL60 cells to differentiate into mature myeloid cells with loss of proliferative capacity to acquire a monocyte-like phenotype (5). As hematopoietic stem cells show lower stage of differentiation in various lineages, their immunophenotype changes could be analyzed by different markers. In leukemic cells, the anti-proliferative capacity was accompanied by a marked increase in the expression of differentiation markers such as CD14 or CD11b (6). VD₃ binds to a vitamin D receptor (VDR) and to an uncharacterized membrane receptor that interacts with protein kinase C (PKC) and activates mitogen activated protein kinases (MAPKs) (7). Several studies have shown that HL60 cell differentiation requires the involvement of complex systems such as enzymatic translocation and activation (phospholipase C, phosphatidylinositol 3-kinase, protein kinase C). VD₃ effects involve fast activation pathways of kinase proteins especially towards the differentiation process of keratinocytes and myeloid cells.
Among synthesized analogs of VD₃, Seocalcitol (EB1089) is ~50-200 times more potent than VD₃ in most cancer cells with respect to inhibition of cell proliferation. This analog shows strong anti-proliferative and differentiating activities on cancer cells (1,2).

Several antineoplastic agents generate and release free radicals that cause cellular damage. Redox cycling leading to oxidative stress has been proposed as one of the mechanisms by which different agents cause damage, particularly at the DNA level and exert their cytotoxicity in different cancer cells (8). The final damage occurring in target cells will depend on the rate of ROS formation and on the efficiency of cellular defense mechanisms. The proliferative phenotype is essential for the cell response towards oxidative stress (9). VD₃ or EB1089 displayed a high disposal of oxidant features and consequently the ability to cause release of reactive oxygen species (ROS) (10,11). Non-toxic doses of VD₃ could induce active cell death within oxidative stress mechanism, via generation and release of ROS in MCF-7 cells. Our previous study has described the pro-oxidant activities of VD₃, and EB1089 and the relationship between oxidative stress induced by these two agents and cellular autofluorescence (12).

Doxorubicin (Dox) is a chemotherapeutic agent useful in treating various cancers. Dox is a quinone-containing anthra-cycline chemotherapeutic and is known to produce ROS in the heart (13). VD₃ enhances the susceptibility of breast cancer cells to doxorubicin-induced oxidative damage (14). This treatment can induce serious side-effects in various tissues, including brain, in addition to known cardiotoxic effects, which limit its successful use in chemotherapy.

Antioxidant supplementation to anticancer drugs decreases oxidative DNA damage in human cells. N-acetyl-cysteine (NAC), superoxide dismutase (SOD) and catalase (CAT) are strongly involved in cellular defences against the oxidative damage caused by anticancer drugs. NAC is thought to act by raising intracellular concentrations of GSH and by the direct scavenging of ROS (15,16). SOD represents the first line of defense of the cellular antioxidant system against the oxidative damage mediated by superoxide radicals, removing superoxides by catalysing the dismutation of two superoxide radicals to yield hydrogen peroxide (H₂O₂). This ROS is finally transformed into H₂O and oxygen by CAT (14).

As regards the myeloid differentiation and the ROS generation, this study aimed to compare VD₃ and EB1089 activities. To enhance differentiation and to limit ROS generation, deltanesoids were supplemented with doxorubicin and antioxidants. These association results determined whether oxidative stress and monocyte differentiation could be dissociated.

Materials and methods

Cell lines and culture conditions. Human promyelocytic HL60 leukemia cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). These HL60 cells were grown in a 5% CO₂ atmosphere at 37°C in RPMI-1640 (1X) medium (Bio-Wittaker, France) with L-glutamin, supplemented with 15% fetal calf serum (FCS) (Gibco-BRL, UK). Cell growth and viability were assessed utilizing the trypan blue exclusion technique (Sigma, France). Cells were routinely examined for mycoplasma contamination.

Chemicals. VD₃ was purchased from Sigma. EB1089 was a generously gift provided by Dr Lise Binderup from Leo Pharmaceuticals Products (Ballerup, Denmark). VD₃ was diluted in absolute ethanol at 10 μM stock solution. EB1089 was supplied as solution diluted in isopropanol (2-propanol) at 4x10⁻⁴ M. Dilutions were performed in absolute ethanol to give stock solutions of 100 μM. The aliquots of stock solutions from both compounds were stored at -20°C, protected from light. The final ethanol concentration was 0.1% in the culture medium and had no effect on either cell growth or oxidative stress. Doxorubicin (Dox), used as hydrochloride, was prepared as 10⁻¹ M stock solution (R. Bellon, France) in distilled water and stored at -20°C. N-acetyl cysteine (NAC), superoxide dismutase (SOD) and catalase (CAT) were purchased from Sigma. NAC was dissolved in phosphate buffer saline (PBS) 1X to form a 1 M stock solution and the pH was adjusted to 7.3 with sodium hydroxide solution. Both SOD and CAT enzymes were also dissolved in PBS 1X to get stock solutions at 10⁻⁴ U/ml and 15x10⁻⁳ U/ml, respectively.

All antioxidant agents were freshly prepared and added to the cell culture medium 1 h before VD₃ or EB1089 incubation.

Determination of cell differentiation. HL60 cell differentiation was assessed by nitroblue tetrazolium reduction assay (NBT). This assay is based on the ability of mature cells (differentiated cells) to reduce tetrazolium salts into formazan beads, due to the superoxide anions they produce upon stimulation with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma). To measure the NBT reduction, 2x10⁵ cells were harvested by two centrifugations (400 g, 10 min) and incubated with 200 μl solution containing RPMI-1640, FCS, 1% NBT (Sigma) and 100 nM TPA for 30 min at 37°C in the dark. Then, cells were examined by optical microscopy. The percentage of purplish cells, indicative of formazan beads presence, was assessed in comparison with untreated cells. At least 300 cells were counted for each experiment.

Measurement of ROS release by fluorescence spectroscopy. The 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) probe (Molecular Probes, France) has the ability to detect ROS production in situ. The cell-permeant form, H₂DCF, rapidly diffuses through the cell membrane and then is hydrolyzed by intracellular esterases to an oxidative-sensitive form, dichlorodihydrofluorescein (H₂DCF). In the cytosol, oxidation of H₂DCF leads to a fluorescent compound, dichlorofluorescein (DCF) with a fluorescence intensity proportional to intracellular ROS (16). H₂O₂, a well known agent for its oxidative property, was used as a positive marker of oxidative stress.

This fluorescent dye allowed to determine ROS release in HL60 cells, untreated or treated VD₃, or EB1089. Briefly, after treatment, HL60 cells were washed and resuspended at 10⁶ cells/ml in RPMI-1640 without FCS and phenol red. Then, 10 μM H₂DCFDA probe was added to each plate at a final volume of 2 ml. Cells were incubated for 45 min at 37°C.
in the dark. A second wash was made before the fluorescence analysis using spectrometer at 488 nm intensity excitation $\lambda_{ex}$ and 516 nm emission $\lambda_{em}$. Results, in arbitrary fluorescence units (AFU), were expressed according to the ratio [(AFU-treated cells)/(AFU control cells)] x100.

Statistical analysis. The data, presented as mean ± SD, were obtained from at least three independent experiments. Significant differences were determined using the Student's t-test (p<0.05 was considered to indicate significant differences).

RNA extraction and RT-PCR analysis. HL60 cells were exposed to the following treatments: VD$_3$ (1-100 nM); EB1089 (0.1-10 nM) and antioxidant agents (NAC 10 mM, CAT 2000 U/ml, SOD 50 U/ml) for the indicated times. Total RNAs from 5x10$^6$ cells were isolated using Tri Reagent™ (Sigma). For RT-PCR, aliquots (1 μg) of total RNA were incubated with 2 μl of a mix containing 10 mM dNTP (Boehringer, France), 2 μl buffer 10X, 1 μl of a 500 μg/μl random hexadeoxynucleotide primers (RH), 4 μl of 25 mM MgCl$_2$, 0.5 μl of human placenta ribonuclease inhibitor (HPRI: Amersham, France) at 40 U/μl plus 0.42 μl of 24 U/μl Moloney murine leukemia virus reverse transcription (M-MLV reverse transcriptase, Gibco-BRL, France) and RNase/DNase-free water to get a final volume of 20 μl. The samples were kept for 10 min at room temperature, then incubated 1 h at 42°C and 5 min at 95°C. RNAs were transcribed into cDNA which were stored at -20°C, after adding 180 μl of DNAse/RNase-free water. PCR were performed with 1/20 volume of the reverse transcription reaction for amplification. These amplifications were performed in a total volume of 50 μl containing 5 μl of buffer 10X, 1 μl of 10 mM dNTP, 4 μl of 25 mM of MgCl$_2$, 28.8 μl of DNase/RNase-free water, 5 U/μl of Taq DNA polymerase (Invtrogen Life Technologies, France) and 1 μg/μl of primers specific to CD14 and GAPDH genes as follows: CD14 (5'-TAAAGGAC TGCCAGCCAAGC-3' and 5'-AGCCAAGGCAGTGTGAG TGC-3'), GAPDH (5'-CTCTGGCCCCCTCTGATGC-3' and 5'-CCATACGCGCCACATTTGGC-3'). Amplification profiles included denaturation at 95°C for 30 sec, primer annealing at 58°C for 30 sec, and extension at 72°C for 30 sec. All the amplifications were performed with 30 cycles for CD14 and GAPDH. After PCR, reaction mixtures were analyzed by a 2% agarose gel electrophoresis. In order to ensure total absence of any contamination negative controls, no RNA and no cDNA, were included in the RT-PCR reactions.

Determination of surface antigen by flow cytometry. HL60 cells, treated or not with VD$_3$ or EB1089 were incubated at various indicated times. Aliquots of 10$^6$ cells were collected to determine CD14 and CD71 expression by flow cytometric analysis. Cells were washed once in PBS and re-suspended in 200 μl of PBS 1X (supplemented with 2% of SVF and 0.01% NaN$_3$). Cell suspensions were gently mixed, protected from light, with 200 μl of Mo2-fluorescein isothiocyanate conjugated (FITC) anti-CD14 (Beckman Coulter, France) or 40 μl of YDJ1.2.2.-Phycocerythrin (PE) conjugated anti-CD71 and incubated for 30 min at 4°C. For each 10$^6$ cell density, 5 μl of anti-CD14 or MsIgM solution stock was diluted in 195 μl PBS 1X.

After incubation, the cells were washed twice in PBS and re-suspended in 500 μl PBS 1X containing 1% paraformaldehyde and stored at 4°C, always protected from light. As negative controls, FITC-labeled MsIgM for CD14 and PE-labeled IgG1 for CD71 were used to set threshold parameters. In each sample, at least 10000 cells were collected and analyzed by a FACS Calibur flow cytometer (Becton Dickinson, France).

Results

Modulation of functional differentiation. VD$_3$ and EB1089-induced differentiation of HL60 cells into monocytic lineage was studied by expression of mRNA, protein CD14 and functional differentiation using nitroblue tetrazolium assay (NBT). The effects of VD$_3$ and EB1089 were studied on HL60 cell differentiation, using the NBT assay. Fig. 1 displays the percentage of positive NBT (NBT$^+$)-treated cells, by increased concentrations of VD$_3$ (1-100 nM) or EB1089 (0.1-10 nM). VD$_3$ and EB1089 induced cell differentiation in a dose-dependent manner. A higher differentiating activity was observed for 1 nM EB1089 than for 1 nM VD$_3$. We examined
the doxorubicin capacity to increase the differentiation activity of VD₃ or EB1089. HL60 cells were incubated with 15 nM doxorubicin for 1 h before a continuous exposure with VD₃ (1-100 nM) or EB1089 (0.1 nM) for 72 h. The percentage of NBT⁺ cells was unaffected by doxorubicin when associated with VD₃, as compared with VD₃ alone (Fig. 1). On the contrary, for 0.1 nM EB1089-treated cells, 15 nM doxorubicin induced an increase of NBT⁺ cell percentage from 29% to 87%. Note that 15 nM doxorubicin exposure has no effect on cell differentiation and viability. VD₃ or EB1089 were also associated with 10 nM NAC, 50 U/ml SOD or 2000 U/ml CAT (Fig. 2). Compared to VD₃ and EB1089 treatments, these associations induced a slight increase of NBT⁺ cells. The highest effect occurred in the case of the EB1089-NAC association. Each antioxidant alone induced no growth inhibitory effect and <3% of NBT⁺ cells (data not shown).

Modulation of mRNA CD14 and CD14 antigen expression.

The mRNA CD14 gene expression was analyzed during HL60 monocytic differentiation. During treatments with VD₃ or EB1089, mRNA levels for ubiquitary transcripts GAPDH did not vary significantly. After total RNA isolation and RT-PCR, mRNA CD14 levels were slight for untreated cells and 1 nM VD₃ (Fig. 3A). A significant increase of 40- and 50-fold in CD14 gene expression was observed for 10 and 100 nM VD₃ (Fig. 3A). The lowest 0.1 nM concentration of EB1089 induced a marked CD14 gene expression which was 40-fold higher than control cells (Fig. 3B). This result confirms the higher differentiating potential generated by EB1089, as compared with VD₃.
The modulation of mRNA CD14 gene expression by NAC, SOD and CAT antioxidants is presented on Fig. 4. After a 72 h incubation of VD3 or EB1089 with antioxidant agents (10 nM NAC, 50 U/ml SOD or 2000 U/ml CAT), CD14 gene expression showed a modulation of mRNA. Semi-quantification displays a significant increased expression of mRNA CD14 in co-treated cells (Fig. 4). In the absence of VD3 or EB1089, each antioxidant was unable to stimulate significantly CD14 gene expression, as compared to untreated cells (data not shown). Moreover, cells were treated with VD3 or EB1089, associated with 15 nM doxorubicin. The EB1089-doxorubicin association induced a significant increase of mRNA expression, as compared with EB1089 (0.1 nM) (Fig. 4B).

After a 72 h exposure to VD3 or EB1089, the induction of the CD14 monocyte marker to immature HL60 cells was studied by flow cytometry (Table I). In the absence of a differentiation stimulation, only 2% of cells were CD14 +. The VD3 treatment induced 12% of CD14 + cells, that increased significantly to 19% and 20% when associated with NAC and SOD antioxidants, respectively.

Modulation of ROS release. It has been well described that some differentiation inducing agents such as VD3 and the EB1089 analog display oxidant features and consequently ROS release. Evaluation of ROS was performed by the H2-DCFDA fluorescent dye, whose emission intensity was expressed as a percentage of control cell intensity. VD3- or EB1089-treated HL60 cells showed an increase of H2DCFDA fluorescence intensity (p<0.05), that corresponds to an oxidative effect.

The effects of NAC, SOD, CAT antioxidants to modulate ROS release in VD3- or EB1089-treated cells was investigated (Fig. 5). Results are expressed as H2DCFDA fluorescence intensity, with respect to untreated cells (100%). Cells were pre-treated for 1 h with each antioxidant before addition of VD3 or EB1089. Antioxidants induced a decrease (p<0.05) in ROS release generated by VD3 or EB1089 near the level of untreated cells. This suggests a protective role of these antioxidant agents against VD3 and EB1089 oxidative cytotoxicity.

HL60 were treated with 15 nM doxorubicin to induce oxidative stress (Fig. 5). We investigated a possible additive effect of doxorubicin in combination with VD3 or EB1089, in

<table>
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<th>Antioxidant supplementation</th>
<th>VD3, 10 nM CD14 + (%)</th>
<th>EB1089 0.1 nM CD14 + (%)</th>
<th>Control CD14 + (%)</th>
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<td>12±2</td>
<td>25±2</td>
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<tr>
<td>CAT 2000 U/ml</td>
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<td>25±4</td>
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<td>NAC 10 mM</td>
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<td>1±1</td>
</tr>
<tr>
<td>SOD 50 U/ml</td>
<td>20±3*</td>
<td>30±5</td>
<td>1±1</td>
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HL60 cells were treated with each antioxidant for 1 h and then with VD3 (10 nM) or EB1089 (0.1 nM) for 72 h. Cell differentiation was determined by percentage of cell expressing the membrane antigen CD14 (CD14 +), as determined by flow cytometry. Values correspond to the average of 3 separate experiments (Mean ± SD). *p<0.05, significantly different with no antioxidant supplementation (Student’s t-test).

Figure 5. Oxidative stress induced by VD3 (A) or EB1089 (B) supplemented with antioxidants or doxorubicin. HL60 cells were treated to 10 mM NAC, 50 U/ml SOD, 2000 U/ml CAT or 15 nM doxorubicin. Cells were labelled with H2DCFDA (10 μM) for 45 min. The fluorescent intensity is shown as the percentage of untreated cells. Results correspond to the average of 3 separate experiments (Mean ± SD).
modulating oxidative stress. The association with doxorubicin induced a significant increase of ROS release (p<0.05) versus 1 nM VD₃ and 0.1 nM EB1089-treated cells (Fig. 5). An additive ROS release (p<0.05) was observed in comparison with individual effects of doxorubicin and VD₃.

Discussion

The comparison of VD₃ with its analog has shown that EB1089 was more effective than the native hormone with 10-100 lower concentrations. Similar results have been described for U937 monocyte cells, MCF-7 carcinoma cells and HaCaT keratinocytes (17,18). These results are due to enhanced effects of EB1089 to cell cycle regulation through c-myc, c-fos, p21 and p53. In MCF-7 cancer cells, EB1089 induced a higher decrease of estrogen receptors than VD₃ (19). According to the drug used, HL60 differentiates either to granulocyte, monocyte or macrophage lineages. In a first step, HL60 cell differentiation was appraised by using the NBT test. The number of differentiated cells (NBT⁺) increases for 48 h of the treatment. To detail VD₃ and EB1089 properties, the NBT test was completed by CD14 expression. CD14 antigen was highly expressed by monocyte and macrophage lineages. Following the treatment with 10 nM VD₃ or 0.1 nM EB1089 for 72 h, the mRNA CD14 and CD14 expressions strongly increased, associated with the abolishment of CD71 expression (data not shown). A time course study demonstrated that mRNA CD14 expression occurred after 6 h VD₃ treatment, whereas CD14 expression and NBT⁺ cells were not observed after this time period.

VD₃ and EB1089 present oxidative properties displayed by ROS release which is dose- and time-dependent. We confirm that one biological mechanism of VD₃ and EB1089 is based on ROS induction (11). The comparison of EB1089 and VD₃ activities indicates that EB1089 is as active as VD₃, with 10-100 lower concentrations. VD₃ brought an increase in the cellular redox state as reflected in the ratio between oxidized and reduced glutathione and glyceraldehyde-3-phosphate dehydrogenase. In addition, the expression of the antioxidant enzyme Cu/Zn superoxide dismutase decreased (20). These reactive oxygen species are involved in the anticancer activity of vitamin D on its own and in its cross-talk with other anticancer modalities. It has been described that one mechanism of action leading to apoptosis by anticancer agent is based on ROS generation and release. The effect of VD₃ to the damage inflicted on breast cancer cells has been examined by the direct action of ROS. VD₃ sensitizes to ROS-induced death by affecting both caspase-dependent and -independent modes of cell death upstream to mitochondrial damage (21). This increased oxidative stress by VD₃ was manifested by glutathione depletion and was abolished by exposure to the thiol antioxidant NAC.

Recent evidence suggested that ROS and antioxidant enzymes could be associated with cell differentiation and disease (22). In this study, SOD, CAT, NAC antioxidants were associated with VD₃ and EB1089 to modulate their differentiation activity. After 72 h of exposure, NAC, SOD and CAT slightly increased the percentage of NBT⁺ cells, CD14 expression and mRNA CD14 expression of VD₃ and EB1089 treated cells. These antioxidants highly reduced ROS generated by VD₃ and EB1089 drugs, suggesting a protective role of these antioxidant agents against latter VD₃ and EB1089-induced cytotoxicity. Thus, the modulation of the cell redox status influences the induction of differentiation. The NAC antioxidant, a sulphydryl reductant, reduces oxidised glutathione, as well as being a precursor of intracellular cysteine and glutathione. Moreover, NAC regulates the conversion from proliferation to differentiation at a transcriptional level (23). The addition of antioxidants to VD₃ activated the JNK pathway as indicated by increased phosphorylation of c-jun and ATF-2. The potentiation of differentiation by antioxidants was inhibited by JNK inhibitor SP600125 (24). Thus, the JNK-API pathway has an important role in the potentiation of VD₃-induced differentiation by antioxidants, and regulates expression of Egr-1 and c-fos (25). Antioxidants such as polyphenols, carotenoids, tocopherol and lipoic acid also potentiate myeloid differentiation by VD₃ or ATRA (26). In addition, the antioxidant carnosic acid, a phenolic terpen, increases the potency of VD₃ to subtype JNK pathways. Rather than their various chemical structures, the common property of these antioxidants to decrease ROS would play an important role to modulate differentiation.

In order to potentiate VD₃-induced differentiation, the association with subtoxic doxorubicin concentrations was evaluated. In presence of doxorubicin, differentiation effect (NBT⁺ cells) of EB1089 was clearly enhanced. However, mRNA CD14 and CD14 expressions were not significantly modulated by doxorubicin. VD₃ was associated with other topoisomerase II inhibitors, such as etoposide and mitoxantrone to potentiate more clearly their differentiation activity (27). The modulation of oxidative stress by doxorubicin was also investigated. VD₃ synergistically enhanced the susceptibility of MCF-7 breast cancer cells to doxorubicin and menadione (14,28). In addition, the pre-treatment of cancer cells with VD₃ or EB1089 selectively increased their susceptibility to doxorubicin-induced apoptosis, as indicated by increased expression of Egr-1 and c-fos (29). These results are due to various chemical structures, the common property of these antioxidants to decrease ROS would play an important role to modulate differentiation.

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References


