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Differential apoptotic response of human melanoma cells to 1α,25-dihydroxyvitamin D_3 and its analogues

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Abstract

Pleiotropic actions of the biologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (VD), include antiproliferative effects in both normal human melanocytes and malignant melanoma cell lines. In this study the actions of VD and its low calcemic analogues EB1089 and CB1093, have been examined in two human melanoma cell lines MeWo and WM1341. Both cell lines express similar amounts of vitamin D receptor mRNA and show functional gene regulatory effects in response to VD and its analogues. VD, EB1089 and CB1093 induced apoptosis only in WM1341 cells and not in MeWo cells, even though both cell lines responded well to etoposide, a strong inducer of apoptosis. Additionally, these results were confirmed by analysis of cell morphology. Interestingly in WM1341 cells, CB1093 was found to be more potent in inducing apoptosis than EB1089 and the natural hormone. Moreover, CB1093 appeared to induce apoptosis at a relatively low concentration of 0.1 nM, whereas greater than tenfold higher concentrations of VD and EB1089 were needed to obtain comparable effects. These observations highlight CB1093 as a promising drug for a future treatment against specific types of melanoma.

Keywords: vitamin D_3; vitamin D_3 analogues; melanoma cell lines; apoptosis

Abbreviations: CB1093, 20-epi-22(S)-ethoxy-23yne-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3; CAT, chloramphenicol acetyl transferase; DR3, direct repeat spaced by 3 nucleotides; EB1089, 22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3; IP9, inverted palindrome spaced by 9 nucleotides; TNFα, tumor necrosis factor alpha; VD, 1α,25-dihydroxyvitamin D_3; VDR, 1α,25-dihydroxyvitamin D_3 receptor; VDRE; VD response element

Introduction

The seco-steroid 1α,25-dihydroxyvitamin D_3 (VD), the biological active form of vitamin D_3, has pleiotropic physiological effects (Walters, 1992). The classical function of VD is its role as the central regulator of calcium homeostasis, which is crucial for bone formation (Reichel and Norman, 1989). Moreover, VD has been shown to inhibit cell growth and to induce differentiation in several normal and malignant cell types (Abe *et al*, 1981; Colston *et al*, 1982; Frampton *et al*, 1982, 1983), but recently VD has also been shown to induce apoptosis (programmed cell death) in human breast cancer and leukemic cell lines (Elstner *et al*, 1995, 1996; James *et al*, 1995; Danielsson *et al*, 1997). *In vivo* and *in vitro* studies of these cell regulatory functions of VD demonstrate its promising therapeutic potential (Jones and Calverley, 1993; Pols *et al*, 1994). These positive cell regulatory VD functions are limited by side effects such as hypercalcemia, hypercalciuria and soft tissue calcification (Vieth, 1990), which are caused by the classical function of the nuclear hormone. Therefore, VD analogues with modifications mainly in the side chain have been tested for both their calcemic and their antiproliferative effects. During the last decade over 800 VD analogues have been synthesized in an effort to dissociate the effects on proliferation and differentiation from those on calcium homeostasis (Bouillon *et al*, 1995).

VD and its analogues are lipophilic molecules that easily pass biological membranes and bind with high affinity to the vitamin D receptor (VDR) (Pike, 1991; Walters, 1992), which is a transcription factor belonging to the superfamily of nuclear receptors (Mangelsdorf *et al*, 1995). VDR binds as a dimeric complex to a pair of hexameric core binding motifs, referred to as VD response elements (VDREs) (Carlberg, 1995). The main partner of the VDR is the retinoid X receptor, which is the nuclear receptor for 9-*cis* retinoid acid (Carlberg, 1996). VDR-RXR heterodimers bind to direct repeats separated by 3 nucleotides (DR3) and to inverted palindromes spaced by 9 nucleotides (IP9) (Schräder *et al*, 1995). It was observed that the highly antiproliferative VD analogue EB1089 (Mathiasen *et al*, 1993) activated an IP9-type VDRE at 15-fold lower concentrations than a DR3-type VDRE (Nayeri *et al*, 1995). Recently, the antiproliferative effect of the VD analogue CB1093 on human MCF-7 breast cancer cells and its effects on *in vivo* regression of rat mammary tumors were found to be even superior to those of EB1089, but most interestingly, CB1093 was found to induce apoptosis at a tenfold lower concentration than EB1089 (Danielsson *et al*, 1997). Moreover, in contrast to EB1089, CB1093 shows a clear preference for the induction of DR3-type VDREs (Danielsson *et al*, 1997). *In vivo* EB1089 and CB1093 have calcemic effect of only 50 and 27% that of VD, respectively (Danielsson *et al*, 1997), and are therefore considered as good candidates for therapeutical use against several types of cancer.

The rather ubiquitous expression of the VDR had suggested that the cell regulatory action of VD and its

analogues in a variety of different cell types should be assessed. One of the first types of cancer, in which an antiproliferative effect of VD could be shown, was the human malignant melanoma cell line Hs695T (Colston *et al*, 1981). Subsequently to this initial report, the induction of differentiation of human malignant melanoma cell lines could also be demonstrated (Mason *et al*, 1988). *In vivo* studies suggested that the expression of the VDR is central for obtaining growth suppression of tumor xenografts (Eisman *et al*, 1987) and this view was supported by an *in vitro* study with human malignant melanoma cell lines (Evans *et al*, 1996).

In this study, the two melanoma cell lines MeWo and WM1341 were chosen as representatives of advanced (malignant) and early (benign) stages of melanoma, respectively (Lu and Kerbel, 1993). Although VD signalling is functional in both cell lines, VDR ligands could induce apoptosis only in WM1341 cells suggesting that the expression of VDR is a necessary but not the only criterion for obtaining potent effects of VDR ligands in the treatment of melanoma. Moreover, this study indicates that the VD analogue CB1093 appears most effective in inducing apoptosis in WM1341 cells.

Results

The effects of VD and its two analogues EB1089 and CB1093 (structures of their side chain are shown in Figure 1) on the two human melanoma cell lines MeWo and WM1341 were investigated. Firstly, the relative amount of VDR mRNA expression was compared by using semiquantitative RT-PCR (Figure 2). Both cell lines were found to express relatively equal quantities of VDR mRNA and these expression levels were not significantly modulated by treatment with high concentrations of VD, EB1089 and CB1093. Functionality of VD signalling was then tested by transiently transfecting both melanoma cell lines with a human osteocalcin VDRE-driven chloramphenicol acetyl transferase (CAT) reporter gene construct (Carlberg *et al*, 1993) and treatment with VD, EB1089 and CB1093 (Figure 3). All three VDR ligands were able to induce CAT activity 3.5–5-fold, compared to solvent-treated controls, demonstrating that both cell lines contain all essential components that allow transactivation through the VDR.

MeWo and WM1341 cells were treated with graded concentrations of CB1093 (as a representative of a potent VDR ligand) in order to assess the effect of VDR ligands on growth of these cells. Cell number was determined after 3, 5 and 7 days of treatment (Figure 4). After a treatment of 7 days the cell number was found to be decreased dose dependently in both cell lines, whereas 5 days of treatment resulted in only slight effects and 3 days of treatment in no significant effects. At lower concentrations, CB1093 appeared to be more effective on WM1341 cells, but treatment with CB1093 at a concentration of 10 nM for 7 days resulted in a reduction of cell number down to approximately 50% of solvent-treated controls in both cell lines. In this context, it is important to note that the growth rate of MeWo cells is clearly faster than that of WM1341 cells.

Consequently, both cell lines were compared for the induction of apoptosis by VD, EB1089 and CB1093. After 5 days of treatment the amount of DNA fragmentation was quantified by measuring free cytoplasmatic nucleosomes

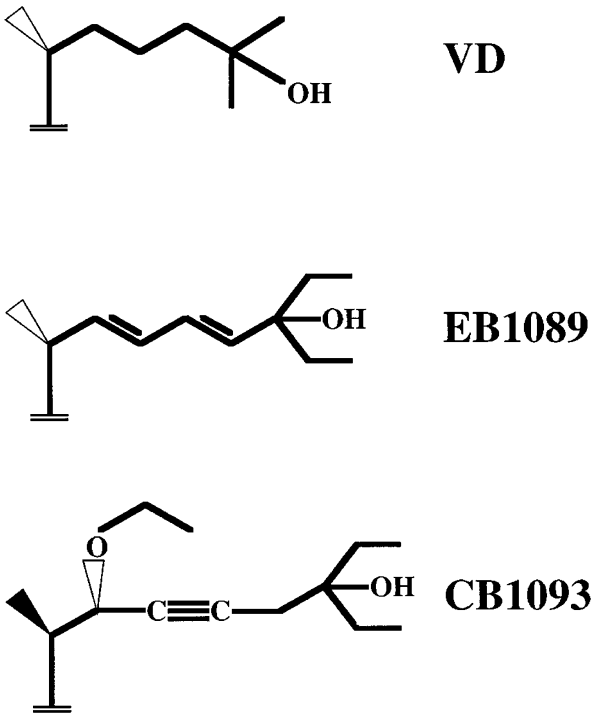

Figure 1 The side chain structure of the VD analogues EB1089 and CB1093 in comparison with VD. The two analogues EB1089 (22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3) and CB1093 (20-epi-22(S)-ethoxy-23yne-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3) were obtained by modifying the side chain of the VD molecule

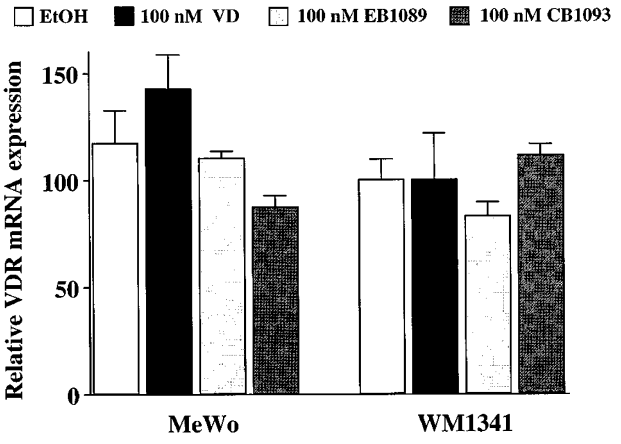

Figure 2 VDR mRNA expression in MeWo and WM1341 cells. MeWo and WM1341 cells were treated with VD, EB1089, CB1093 (all at 100 nM) and 0.1% ethanol (as control) for 2 h. The relative amount of VDR mRNA expression was determined by semiquantitative RT–PCR normalized for β_2-microglobulin ('housekeeping') gene mRNA expression. Columns represent mean values of triplicates; the bars indicate standard deviations

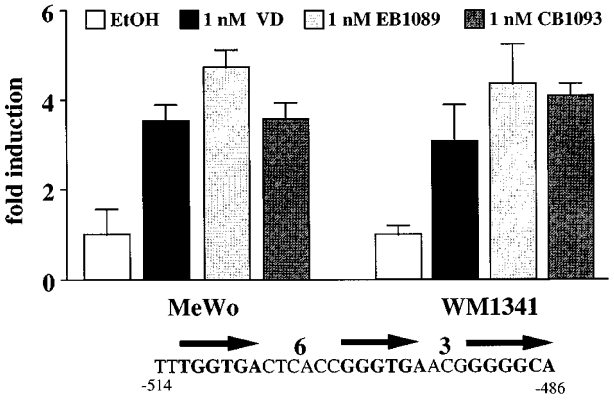

Figure 3 VDRE-driven reporter gene activation in MeWo and WM1341 cells. MeWo and WM1341 cells were transfected with a *tk* promoter/CAT reporter gene construct containing the complex VDRE of the human osteocalcin gene (core sequence indicated below). The cells were treated with VD, EB1089, CB1093 (all at 1 nM) and 0.1% ethanol (as control); CAT activities were determined 40 h later and normalized to β-galactosidase activities. Columns represent mean values of triplicates; the bars indicate standard deviations

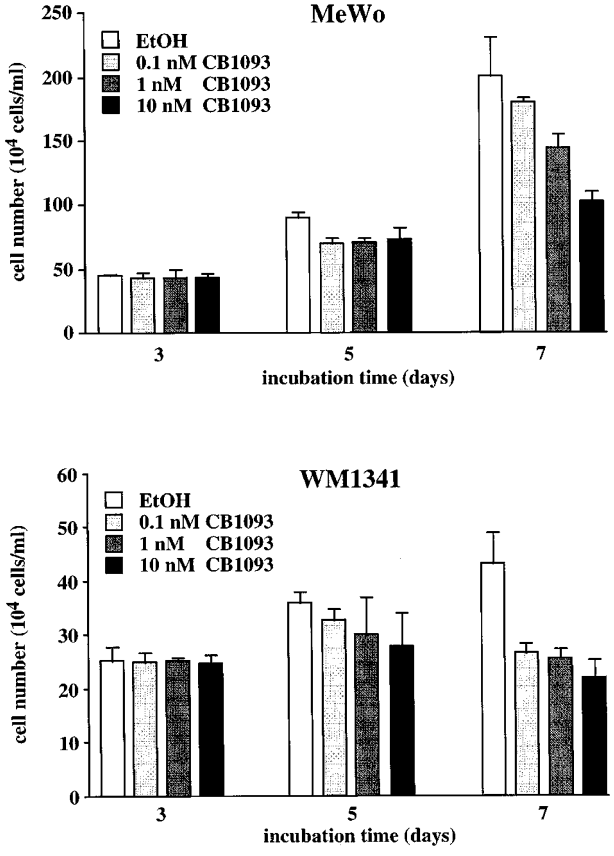

Figure 4 Growth study of MeWo and WM1341 cells. MeWo and WM1341 cells were treated with graded concentrations of CB1093 and cell number was determined after 3, 5 and 7 days. Columns represent mean values of 3–10 determinations, the bars indicate standard deviations

using the Cell Death Detection ELISA assay (Figure 5). Interestingly, in MeWo cells, the VDR ligands tested were unable to induce apoptosis, even at highest concentrations, whereas in WM1341 cells apoptosis was induced up to 20-fold. In the latter cell line, VD and its analogues differed in their potency. Briefly, with CB1093 a half-maximal induction of apoptosis was already obtained at a concentration of 0.09 nM, whereas for EB1089 and VD the half-maximal induction was determined as 0.2 and 1.1 nM, respectively. These results highlight that CB1093 was apparently the most effective compound. Therefore, the induction of apoptosis in WM1341 cells by CB1093 was subsequently followed over a time course of 7 days (Figure 6). After 3 days of treatment with concentrations ranging from 0.1–10 nM an eightfold induction of apoptosis was already observed, which increased up to a 36-fold induction after 5 days of stimulation with CB1093. A further 2 day incubation did not result in a significant, additional effect.

Etoposide and tumor necrosis factor alpha (TNFα), both inducers of apoptosis, were compared with CB1093 within a treatment of 2 days, for their ability to induce apoptosis in MeWo and WM1341 cells (Figure 7). In MeWo cells, etoposide was able to induce apoptosis approximately 66-fold, whereas TNFα and CB1093 did not exert any significant effect. Interestingly, in the slowly growing WM1341 cells, etoposide induced apoptosis only fourfold, whereas with a sixfold induction of apoptosis CB1093 appeared to be more potent. In addition to DNA fragmentation, these results were confirmed by morphological changes observed after Hoechst 33342 staining (Figure 7B and C). In MeWo cells, only etoposide was effective, whereas in the WM1341 cell line etoposide- and CB1093-treated cells showed the typical signs of apoptosis such as cell shrinkage, chromatin condensation and nuclear fragmentation.

Discussion

Malignant melanoma is a tumor derived from the pigmented melanocytes located in the epidermal layer of the skin. The incidence of this malignancy has increased rapidly over recent decades emphasizing the importance of investigations on potential therapeutics. This study highlights the VD analogue CB1093 as a promising drug that induces apoptosis in WM1341 cells after 5 days of treatment with doses of only 0.1 nM, i.e. CB1093 appeared to be effective at tenfold lower concentrations than the natural hormone. Moreover, after only 2 days of treatment of the slowly growing benign type melanoma cell line WM1341, CB1093 appeared to be more potent in inducing apoptosis than the strong apoptosis inducer, etoposide. The high apoptotic potential of CB1093 suggests that this analogue should be most effective for *in vivo* regression of melanoma. CB1093 has previously been shown to cause only 27% of the calcemic effect of VD *in vivo* indicating a low risk of side effects from CB1093 and a potential for clinical use.

Previous studies examining VD effects on melanoma cell lines (Colston *et al*, 1982; Evans *et al*, 1996) suggested that VDR expression is crucial for obtaining an antiproliferative response by VD. This study demonstrated that the

expression of the VDR and even functional VD signalling are not sufficient requirements for obtaining an apoptotic response. The fast growing malignant MeWo melanoma cell line responded well to etoposide after only 2 days of treatment, which indicates that this cell line can undergo apoptosis, whereas 5 days of treatment with VD and its analogues was without an effect. However, the molecular mechanisms of the different responsiveness of the two melanoma cell lines are not known yet. So far, WM1341 cells have only been investigated for cellular markers of a metastatic potential, but not for molecular markers of apoptosis such as p53 mutations and bcl-2 expression (Herlyn, 1990; Schadendorf *et al*, 1996a). For the more intensively studied MeWo cells compiling of a profile of molecular markers has just been started (Schadendorf *et al*, 1996b; Kern *et al*, 1997).

Thus far, only a minority of all primary VD responding genes has been identified and none of these genes have yet been associated with the induction of apoptosis. There are indications that the TNFα gene is a primary VD responding gene (Geilen *et al*, 1997), however in both melanoma cell lines tested here treatment with TNFα protein did not result in a significant effect suggesting that an endogenous upregulation of TNFα would also be ineffective. Moreover, both cell lines showed no responsiveness to Fas-ligand (data not shown). A potential induction of differentiation of the melanoma cells either by TNFα or by VDR ligands has not been investigated.

The observation that treatment with CB1093 reduced growth of both melanoma cell lines, but induced apoptosis in only one of them, supports the idea that the effects of VDR ligands on inhibition of the cell cycle and on induction of apoptosis are mediated by different genes. Moreover, these genes may have different types of VDREs in their regulator regions, e.g. preferentially IP9-type VDREs in cell cycle related genes and DR3-type VDREs in apoptosis related genes. This theory would then be in accordance with previous observations that EB1089 has a preference for the activation of IP9-type VDREs, whereas CB1093 has

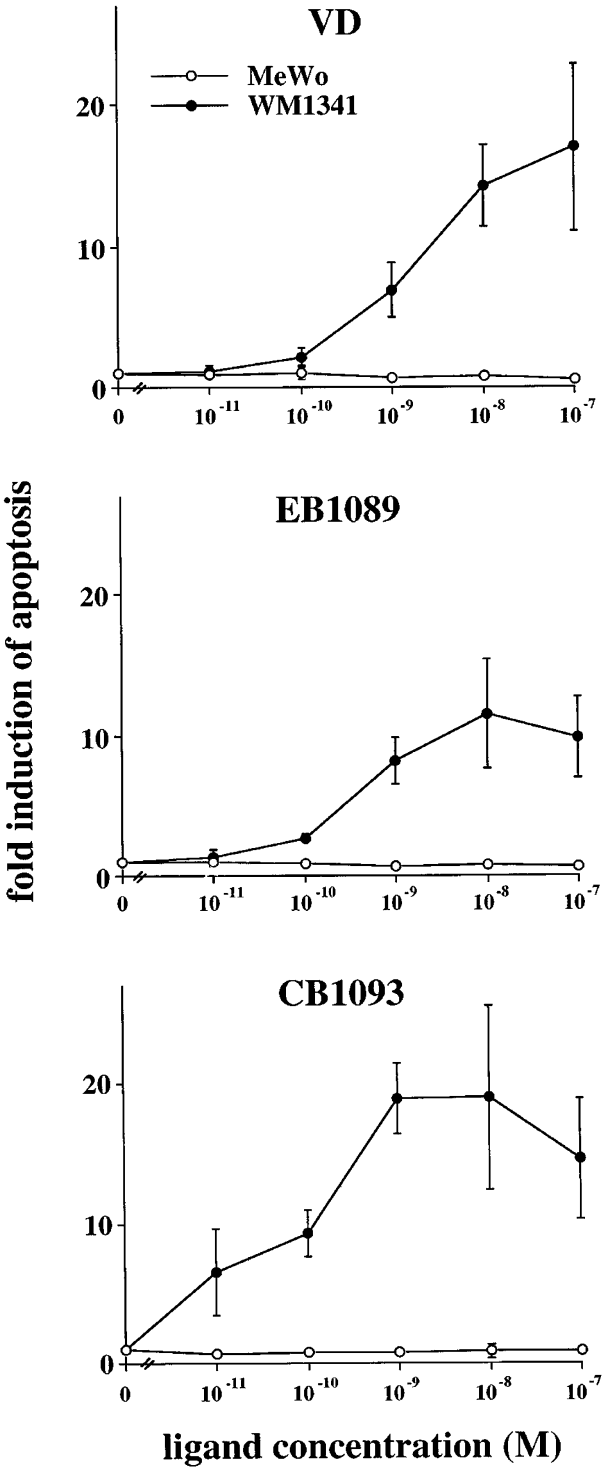

Figure 5 Apoptosis-inducing potential of VD, EB1089 and CB1093. MeWo and WM1341 cells were stimulated with graded concentrations of VD, EB1089 and CB1093 for 5 days. Induction of apoptosis was quantified by the Cell Death Detection ELISA^{PLUS} (Boehringer Mannheim) and normalized by counting cell numbers. Each data point represents the mean of 6–12 determinations and the bars indicate standard deviation

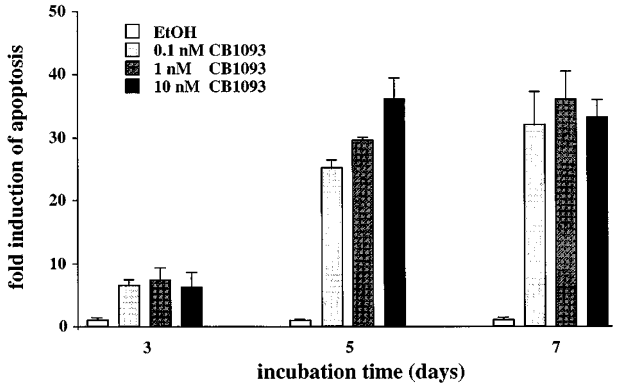

Figure 6 Time-dependent induction of apoptosis by CB1093 in WM1341 cells. WM1341 cells were treated with graded concentrations of CB1093 for 3, 5 and 7 days. Induction of apoptosis was quantified by the Cell Death Detection ELISA^{PLUS} (Boehringer Mannheim) and anormalized by counting cell numbers. Columns represent mean values of 3–6 determinations, the bars indicate standard deviations

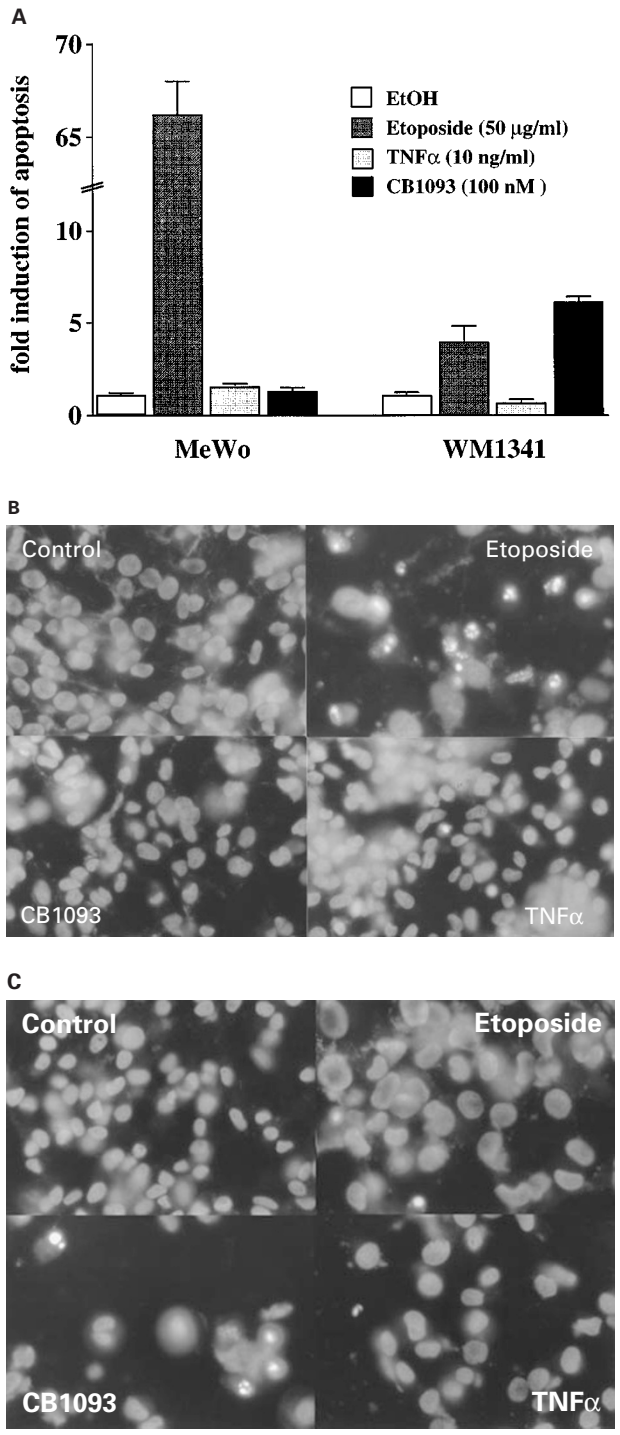

Figure 7 Apoptosis in MeWo and WM1341 cells. MeWo and WM1341 cells were treated with ethanol (0.1%, as control), etoposide (50 μg/ml), CB1093 (100 nM) and TNFα (10 ng/ml) for 2 days. Induction of apoptosis was quantified by the Cell Death Detection ELISA^{PLUS} (Boehringer Mannheim) and normalized by counting cell numbers (**A**). Columns represent mean values of triplicates; the bars indicate standard deviations. Staining with Hoechst 33342 (8 μg/ml) identified MeWo (**B**) and WM1341 (**C**) cells that showed the typical apoptosis associated chromatin condensation and nuclear fragmentation. Representative fields are shown at 400 $\times$ magnification

a selectivity for DR3-type VDREs (Nayeri *et al*, 1995; Danielsson *et al*, 1997). Finally, the difference between MeWo and WM1341 cells in their apoptotic responsiveness provides an interesting model system that may be useful for the identification of the key primary VD responding genes that are involved in the modulation of apoptosis.

In conclusion, a high potency for induction of apoptosis, as shown here for the VD analogue CB1093, is an important prerequisite for an effective tumor regressing potential of an anticancer agent. However for a given cell type, both functional VD signalling and the ability to undergo apoptosis do not appear to be sufficient for predicting apoptotic responsiveness to VDR ligands. CB1093 appeared to be very effective in inducing apoptosis in the early stage WM1341 melanoma cell line, but not in the advanced stage MeWo melanoma cell line. This suggests that treatment of melanoma with VD analogues is effective in the early but not in the late stages of melanoma.

Materials and Methods

Compounds

Comparison of the side chain structures of the VD analogue EB1089 (22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3) to VD shows that EB1089 is elongated by three carbon atoms and contains two additional double bonds (Colston *et al*, 1992). The VD analogue CB1093 (20-epi-22(S)-ethoxy-23yne-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3) has altered stereochemistry at carbon 20 (20-epi analogue) with an ethoxy substitute at carbon 22 and a triple bond at carbon 23-carbon 24 instead of the two double bonds present in EB1089 (Calverley *et al*, 1995). The compounds were synthesized in the Department of Chemical Research (LEO Pharmaceutical Products, Denmark) and dissolved in 2-propanol at a stock of 4 mM.

Cell culture

The human melanoma cell lines MeWo and WM1341 (Herlyn, 1990) were kindly provided by D. Schadendorf and cultured in phenol-red-free RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were seeded in 24 well-plates at a density of 10^4 cells/ml and grown in phenol-red-free RPMI-1640 medium supplemented with 2.5% charcoal-treated FBS for growth studies. VD, EB1089 and CB1093 were added 2 h after seeding and fresh dilutions were added every second day when the medium was changed. Control cells were treated with solvent (0.1% ethanol). Cells were collected for counting after 3, 5 and 7 days of treatment.

Semiquantitative RT–PCR

MeWo and WM1341 cells were cultured in phenol-red-free RPMI-1640 medium supplemented with 2.5% charcoal-treated FBS and at approximately 70% confluence they were treated with VD, EB1089, CB1093 or solvent (0.1 % ethanol) for 2 h. Cells were collected and total RNA was isolated using Trizol (Life Technologies). First strand cDNA synthesis was performed on 2 μg of total RNA using MMLV reverse transcriptase (Life Technologies). 2.5% of this cDNA served as templates for PCR reactions using Taq DNA polymerase (Life Technologies) with a profile of 94°C for 1 min, 58°C for 2 min and 72°C for 30 s for 4 cycles and then 94°C for 10 s, 58°C for 1 min and 72°C

for 30 s for 36 (VDR) or 22 (β_2-microglobulin) cycles, respectively. Primers were 5′-endlabelled using γ[^{32}P]-ATP and T4 polynucleotide kinase (Promega) and had the following sequence:

VDR$^+$ GATGACCCTTCTGTGACCC;
VDR$^-$ AGCTTCTTCAGTCCCACCTG;
β_2MG$^+$CCCCCACTGAAAAAGATGAGTATGCCTG;
β_2MG$^-$ CCTGTGGAGCAACCTGCTCAGATACATC.

Amplified PCR products were separated from un-incorporated primers on a 5% non-denaturing polyacrylamide gel in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.3]), localized by autoradiography, excised and quantified by scintillation counting. The ratio of incorporated to un-incorporated VDR primers was normalized to the respective β_2-microglobulin primer ratio and provides a value for relative VDR mRNA expression.

Transfection and reporter gene assay

MeWo and WM1341 cells were seeded into 6 well-plates (2×10^5 cells per well) and grown overnight in phenol-red-free RPMI-1640 medium supplemented with 5% charcoal-treated FBS. Liposomes were formed by incubating 2 μg of the human osteocalcin VDRE/*tk* promoter/CAT reporter gene construct (Carlberg *et al*, 1993) and 1 μg of the reference plasmid pCH110 (Pharmacia) with 15 μg N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Boehringer Mannheim) for 15 min at room temperature in a total volume of 100 μl. After dilution with 900 μl phenol red-free RPMI-1640 medium, the liposomes were added to the cells. Phenol red-free RPMI-1640 medium (500 μl) supplemented with 15% charcoal-treated FBS was added 4 to 8 h after transfection, at this time ligand was also added. The cells were harvested 40 h after treatment and CAT-assays were performed as described (Pothier *et al*, 1992). The CAT activities were normalized to β-galactosidase activity and induction factors were calculated as the ratio of CAT activity of ligand-stimulated cells to that of mock-induced controls.

Apoptosis assay

Apoptosis was determined by using the Cell Death Detection ELISA^{PLUS} assay (Boehringer Mannheim), which allows quantification of cytoplasmatic histone-associated DNA fragments (mono- and oligonucleosomes). MeWo and WM1341 cells were seeded at a density of 10^4 cells/ml in 6-well plates and grown in phenol-red-free RPMI-1640 medium supplemented with 2.5% charcoal-treated FBS. VD, EB1089 and CB1093 were added 2 h after seeding and fresh dilutions were added every second day when the medium was changed. Control cells were treated with solvent (0.1% ethanol). Adherent cells were harvested by scraping after 3, 5 or 7 days after onset of treatment and assayed according to the manufacturer's instruction (Boehringer Mannheim). Positive controls for the induction of apoptosis cells were grown for 2 days in phenol-red-free RPMI-1640 medium supplemented with 2.5% charcoal-treated FBS in 6-well plates or chamber slides (Life Technologies) in the presence of CB1093 (100 nM), etoposide (50 μg/ml) and TNFα (10 ng/ml). After staining with Hoechst 33342 (8 μg/ml) cell morphology was observed by fluorescence microscopy.

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