Combination of 1α,25-Dihydroxyvitamin D3 with Dexamethasone Enhances Cell Cycle Arrest and Apoptosis: Role of Nuclear Receptor Cross-Talk and Erk/Akt Signaling

Ronald J. Bernardi, Donald L. Trump, Wei-Dong Yu, Terence F. McGuire, Pamela A. Hershberger, and Candace S. Johnson

ABSTRACT

Previously we have shown that dexamethasone (DEX) enhances the antitumor activity and ligand binding of the active form of vitamin D, 1α,25-dihydroxyvitamin D3 (1,25-D3), in the murine squamous cell carcinoma model SCC VII/SF. DEX also reduces the hypercalcemia toxicity of 1,25-D3 treatment. However, the mechanism of the enhanced antitumor activity has not been defined. Here, we demonstrate that both cell cycle arrest and apoptosis were enhanced by DEX, effects that were inhibited by RU486. We also demonstrate that vitamin D receptor (VDR) protein levels were increased by the combination of 1,25-D3 and DEX above the level observed with 1,25-D3 treatment alone, whereas protein levels of the heterodimeric partner of VDR, retinoid X receptor, were lower for the combination than for 1,25-D3 alone. Glucocorticoid receptor protein levels and ligand binding were increased by 1,25-D3 but not by the combination of 1,25-D3 and DEX. Glucocorticoid receptor protein levels and ligand binding were increased by 1,25-D3 but not by the combination. Treatment with the combination of 1,25-D3 and DEX did not result in greater activation of a vitamin D response element-reporter than 1,25-D3 alone or of a glucocorticoid response element-reporter than DEX alone. Nevertheless, the levels of phospho-Erk1/2 and phospho-Akt, signaling molecules that are modulated in 1,25-D3-treated squamous cell carcinoma cells, were reduced by the combination of 1,25-D3 and DEX more than by either agent alone. These trends were also observed in vivo. Our results suggest the involvement of the Erk and Akt signaling pathways in the antiproliferative effects of the combination of 1,25-D3 and DEX and that phospho-Erk1/2 and phospho-Akt may be useful markers of response to this combination.

INTRODUCTION

The active metabolite of vitamin D, 1,25-D3, plays an important role in calcium homeostasis (1). Several laboratories, including our own, have also demonstrated antiproliferative activity for 1,25-D3 in multiple tumor types including squamous cell, prostate, breast, colon, and others (2–9). However, hypercalcemia and hypercalcuria, the dose-limiting toxicities of 1,25-D3, limit its usefulness as a single-agent cancer therapy (10–12). Efforts to develop analogues of 1,25-D3 with less propensity to induce hypercalcemia that maintain antitumor activity are under way (13), but clinical studies indicate that these agents will still cause hypercalcemia (14).

A second strategy that we have used is to combine 1,25-D3 with other agents to develop therapeutic regimens that are both effective and relatively nontoxic. Glucocorticoids are a standard treatment for vitamin D-induced hypercalcemia (15). In addition, glucocorticoids have antiproliferative activity in certain lymphoproliferative disorders and breast cancers as well as palliative effects in patients with prostate cancer (16, 17). We have shown previously that the glucocorticoid DEX significantly reduces 1,25-D3-mediated hypercalcemia and also enhances the antitumor activity of 1,25-D3 in vitro and in vivo in the murine SCCVII/SF SCC model system (18). Although the reduction of vitamin D-induced hypercalcemia by glucocorticoids involves increased urinary excretion of calcium, decreased calcium absorption in the intestine, and potentially other mechanisms (19), the mechanism of enhancement of vitamin D-mediated antitumor activity has not been carefully examined previously and is likely distinct.

Clinical trials are currently in progress to assess the combination of 1,25-D3 and DEX in head and neck SCC and prostate cancer. Preliminary results from a Phase II trial of 1,25-D3 (12 µg qd three times/week) with DEX (4mg qd 4 times/week) in androgen-independent prostate cancer demonstrated a >50% reduction in prostate specific antigen in 5 of 24 evaluable patients (24%) and a decrease in prostate specific antigen velocity in 19 patients (79%; Ref. 20). Mild and transient hypercalcemia was observed in only two patients. Infor

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3 The abbreviations used are: 1,25-D3, 1α,25-dihydroxycholecalciferol; DEX, dexamethasone; SCC, squamous cell carcinoma; VDR, vitamin D receptor; GR, glucocorticoid receptor; RXR, retinoid X receptor; PARP, poly(ADP-ribose) polymerase; VDRE, vitamin D response elements; GRE, glucocorticoid response elements; β-Gal, β-galactosidase; Erk, extracellular signal-related kinase; PI3K, phosphatidylinositol 3-kinase; CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumor virus; qd, once daily.
mation regarding the mechanisms of this combination therapy may provide useful predictors or markers of response and suggest new potential therapeutic targets.

Both 1,25-D$_3$ and DEX are known to bind members of the nuclear receptor superfamily (21). 1,25-D$_3$ functions through the VDR. Binding of 1,25-D$_3$ to the VDR promotes the formation of heterodimers with another member of the nuclear receptor superfamily, the RXR (22). In addition, 1,25-D$_3$ promotes the binding of this complex to specific DNA sequences known as VDRE and the activation of transcription of vitamin D-responsive genes (22–24). Similarly, DEX acts via GR homodimers and GRE (21, 25).

Although most of the activity of nuclear receptors is regulated through the binding of ligand, several examples of crosstalk between nuclear receptors have been demonstrated in which the addition of the ligand of one receptor can affect signaling through another receptor. Such interactions are known to occur via a variety of mechanisms, including transcriptional repression of the estrogen receptor gene by 1,25-D$_3$ and inhibition of estrogen receptor binding to DNA by 1,25-D$_3$, or all-trans-retinoic acid (24, 26, 27). Many nuclear receptors are also known to share coactivators and corepressors (28, 29). In fact, interactions between 1,25-D$_3$ and glucocorticoids have been observed in a variety of tissues; up-regulation or down-regulation of VDR by glucocorticoids is observed depending on the tissue and species examined (30, 31). Furthermore, we have shown previously that DEX significantly increases 1,25-D$_3$ ligand binding in SCC cells (18). Thus, we hypothesized that cross-talk may occur between VDR and GR in cancer cells and that DEX may enhance the antiproliferative effects of 1,25-D$_3$ by increasing the levels of VDR protein and the expression of vitamin D target genes. Although the target genes that are critical for the antiproliferative effects are as yet undetermined, increased expression of such targets could potentially enhance the antitumor activity of 1,25-D$_3$. This hypothesis is consistent with our previous finding that DEX alone has little antiproliferative effect in the SCC model (18).

Several previous studies have provided information regarding the mechanism of the antiproliferative effects of 1,25-D$_3$. 1,25-D$_3$ induces G$_0$/G$_1$ arrest and apoptosis to varying degrees, depending on the cell type (32). Both of these effects have been observed in SCC cells (33, 34). In addition, we have recently reported that 1,25-D$_3$ causes a reduction in both phospho-Erk1/2 and phospho-Akt, particularly in a subpopulation of SCC cells that detach and are apoptotic (34). Both of these molecules are key components of proliferation and survival pathways (35, 36). Others have shown that DEX also inhibits the Erk and Akt signaling pathways in certain cell types (37, 38). In this study, we examined whether the combination of 1,25-D$_3$ and DEX enhances some or all of these changes that are observed with 1,25-D$_3$ treatment.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** The active metabolite of vitamin D, 1,25-D$_3$, was a gift from Milan R. Uskokovic (Hoffmann-LaRoche, Nutley, NJ). It was reconstituted in ethanol, stored under a layer of nitrogen gas at -70°C, and handled with indirect lighting only. DEX 21-phosphate (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water to 25 mg/ml. A 10 mM stock solution of mifepristone (RU486; Sigma Chemical Co.) was prepared in ethanol. 1,25-D$_3$, DEX, and RU486 were diluted to the appropriate final concentrations in tissue culture media immediately before use.

**Tumor Cells and Model System.** The SCC VII/SF murine SCC model, a spontaneously arising tumor of the C3H mouse (39), was maintained in vitro in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 14% inactivated FCS (HyClone, Logan, UT) and 1% penicillin-streptomycin sulfate (Life Technologies, Inc.). Tumors were produced in vivo by s.c. injection in the flank of C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME). Treatment was initiated when tumors were ~100 mm$^3$.

**Crystal Violet Assay.** Antiproliferative effects were assessed using a crystal violet assay essentially as described previously (2). Briefly, SCC cells were seeded in 96-well plates. Various concentrations of DEX and/or RU486 or media were added 24 h later. The next day, various concentrations of 1,25-D$_3$ or media were added. Forty-eight h after the addition of 1,25-D$_3$, the cells were harvested and stained with crystal violet. After drying for 24 h, 100 μl of 33% acetic acid was added to each well, and the absorbance was measured at a wavelength of 540 nm using an ELISA reader (model EL-340; Bio-Tek Instruments, Winooski, VT). Drug interactions were quantitated by median-dose effect analysis, and combination index values were derived as described previously (40) using CalcuSyn software (Biosoft, Ferguson, MO).

**Treatments and Preparation of Whole Cell Lysates.** For in vitro studies, SCC cells were seeded in T25 flasks and allowed to reach confluence. Cells were plated at a cell density that remained subconfluent until the time of harvest, and all treatment groups were performed in duplicate. At various time points, whole cell lysates were prepared as described previously using lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris (pH 8.0), 150 mM NaCl, 0.6 mM PMSF, and 5 μg/ml leupeptin; Ref. 41]. For in vivo studies, mice were treated with 9 g/mouse for 3 days of 1,25-D$_3$ (beginning on the second day of DEX treatments) or saline control. Treatments were given by i.p. injection in 0.2 ml. Mice were treated 6 h after the final treatment and flash-frozen in liquid nitrogen. Lysates were prepared in lysis buffer as described previously (41). Protein concentrations were determined for in vitro and in vivo samples using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

**Western Blot Analysis.** Protein samples were resolved by SDS-PAGE, and transferred overnight to polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA). Western blots were performed as described previously (41). Antibodies used include: rabbit polyclonal anti-active caspase-3 (Alexis Biochemicals, San Diego, CA; 1:200); mouse monoclonal anti-PARP (C2.10; Enzyme Systems Products, Livermore, CA; 1:7500); polyclonal rabbit anti-VDR (C-20; Santa Cruz Biotechnology; 1:400); polyclonal rabbit anti-RXRα (D-20; Santa Cruz Biotechnology; 1:200); polyclonal rabbit anti-human GR (Affinity Bioreagents, Inc., Golden, CO; 1:200); mouse monoclonal anti-phospho-Erk (E-4; Santa Cruz Biotechnolog-
technology; 1:1000); polyclonal rabbit anti-phospho-Akt (Ser473) (Cell Signaling Technology, Beverly, MA; 1:1000); and mouse monoclonal anti-actin (Oncogene Research Products, Boston, MA; 1:6000). Anti-rabbit (Amersham Life Science, Arlington Heights, IL; 1:2500), anti-mouse (Amersham Life Science; 1:5000), and antimouse IgM (Oncogene; 1:6000) horseradish peroxidase-conjugated secondary antibodies were used. Densitometric analyses of Western blot data were performed using a Molecular Dynamics personal densitometer equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Ligand-binding Assay. Analysis of GR binding was measured by a modification of the whole-cell binding assay (42). Briefly, SCC cells were grown in vitro and either treated for 24 h with 4 nM 1,25-D$_3$ or left untreated. Cells were harvested and suspended in medium at a density of $10^7$ cells/ml. Then they were incubated with 50 nM of $[^3]$HDEX (specific activity, 80 Ci/mmol) for 1 h at 37°C with or without a 200-fold excess of unlabeled DEX and harvested by centrifugation. Cell pellets were washed three times in 3.0 ml of HBSS and then suspended in 1.6 ml of HBSS. Cell suspensions (0.5 ml) were added to 5 ml of scintillation fluid, and the radioactivity was counted using a Beckman 5801 liquid scintillation counter. A separate aliquot was used to determine the protein concentration. Nonspecific binding (determined from samples with excess unlabeled DEX) was subtracted from total binding (determined from samples without unlabeled DEX) to determine the specific binding of ligand to GR. The specific binding was then converted from dpm to fmol/mg cellular protein using the specific activity of the $[^3]$HDEX. The mean ± SD of three separate experiments is shown.

Reporter Assays. SCC cells were plated at 1 × 10$^5$ cells/60 mm Petri dish, which allowed them to remain subconfluent throughout the experiment. The next day, the cells were transfected with 5 µg of a VDRE reporter plasmid, (DR+3')$_2$tk-CAT (a gift from Leonard Freedman, Memorial Sloan-Kettering, New York, NY), containing two copies of the VDRE from the mouse osteopontin (Spp-1) gene, or a GRE reporter plasmid, MMTV LTR-CAT (a gift from Don DeFranco, University of Pittsburgh, Pittsburgh, PA), which is known to contain a GRE (43), using the GenePORTER Transfection Reagent system (Gene Therapy Systems, San Diego, CA). The cells were cotransfected with 2 µg of pCMV-SPORT-β-Gal plasmid (Life Technologies, Inc., Rockville, MD) as a control for transfection efficiency. Fresh media containing the appropriate concentration(s) of drug(s) was added 5 h after the addition of the transfection mixture. The cells were then incubated for 48 h and harvested in reporter lysis buffer following the protocol described for the β-Gal Enzyme Assay System (Promega, Madison, WI). Lysates were stored at −70°C. The Quan-T-CAT assay system (Amersham Pharmacia Biotech, Piscataway, NJ) was used to determine CAT activity levels for each group. CAT activity was normalized to β-Gal activity, which was assayed following the protocol of the β-Gal Enzyme Assay System (Promega). The values for the VDRE reporter assay were normalized so that 1,25-D$_3$ equaled 100%, whereas the values for the GRE reporter were normalized so that DEX equaled 100%.

Cell Cycle Analysis. The effect of 1,25-D$_3$ and DEX on cell cycle distribution was assessed essentially as described by Vindelov et al. (44). Briefly, SCC cells were plated and treated as above. Cells were harvested 24 h after treatment and then processed and stained with propidium iodide, filtered through 30-µm nylon mesh, and analyzed by flow cytometry using a FACStar machine (Becton Dickinson, Franklin Lakes, NJ). Cell cycle analysis was computed using the sum of broadened rectangles model with the DNA software from Becton Dickinson.

RESULTS

DEX Enhances Cell Cycle Arrest and Apoptosis Induced by 1,25-D$_3$, and These Effects Are Blocked by RU486. In a previous report, we demonstrated that DEX enhances the antiproliferative effects of 1,25-D$_3$, SCC cells were treated with various concentrations of 1,25-D$_3$ for 48 h with or without pretreatment for 24 h with DEX and/or RU486. A constant ratio of 1:50:250 (1,25-D$_3$: DEX: RU486) was used. The antiproliferative effects were then determined using a crystal violet assay. Results are shown as the percentage of inhibition compared with untreated control cells. Bars, mean of 4 wells ± SD. ∗, where the combination of 1,25-D$_3$ and DEX was significantly different from 1,25-D$_3$ treatment alone (P < 0.05; Student’s t test).

Fig. 1 RU486 inhibits the ability of DEX to enhance the antiproliferative effects of 1,25-D$_3$. SCC cells were treated with various concentrations of 1,25-D$_3$ for 48 h with or without pretreatment for 24 h with DEX and/or RU486. A constant ratio of 1:50:250 (1,25-D$_3$: DEX: RU486) was used. The antiproliferative effects were then determined using a crystal violet assay. Results are shown as the percentage of inhibition compared with untreated control cells. Bars, mean of 4 wells ± SD. ∗, where the combination of 1,25-D$_3$ and DEX was significantly different from 1,25-D$_3$ treatment alone (P < 0.05; Student’s t test).
reduction in the percentage of cells in S phase. This effect was enhanced either or both of these possible outcomes. As has been shown between cell cycle and apoptotic effects, we tested whether DEX combination of 1,25-D$_3$ and DEX caused a greater G$_0$/G$_1$ accumulation compared with 1,25-D$_3$ alone (matched pairs test; Table 1). The combination of 1,25-D$_3$ with DEX also led to an increase in the cleaved, active form of caspase-3, and an additional reduction of full-length PARP as shown (Fig. 2).

RU486 is antagonistic to the combination of 1,25-D$_3$ and DEX (combination index = 2.34 ± 0.21 at the ED$_{50}$).

Because the crystal violet assay does not distinguish between cell cycle and apoptotic effects, we tested whether DEX enhanced either or both of these possible outcomes. As has been reported in SCC and other cell types (32, 33), 1,25-D$_3$ treatment induced an accumulation of SCC cells in G$_0$/G$_1$ phase with a reduction in the percentage of cells in S phase. This effect was observed after treatment with 10 nM 1,25-D$_3$ for 24 h. The combination of 1,25-D$_3$ and DEX caused a greater G$_0$/G$_1$ accumulation compared with 1,25-D$_3$ alone (P < 0.05, Wilcoxon matched pairs test; Table 1). The combination of 1,25-D$_3$ with DEX also led to an increase in the cleaved, active form of caspase-3, and an additional reduction of full-length PARP as compared with treatment with 1,25-D$_3$ alone (Fig. 2); therefore, DEX also enhanced the apoptotic response to 1,25-D$_3$. In addition, the enhancement of cell cycle arrest and apoptosis by DEX were both blocked by the GR antagonist, RU486 (Table 1 and Fig. 2).

The Combination of 1,25-D$_3$ and DEX Increases VDR, but not RXR, Protein Levels. We demonstrated previously that DEX increases 1,25-D$_3$ ligand binding 2.6-fold compared with 1,25-D$_3$ alone (18); therefore, we examined VDR protein levels by Western blot to determine whether an increase in VDR protein correlates with the ligand-binding data. SCC cells were harvested at 4 h and 48 h after treatment, and equal amounts of protein were used for immunoblotting with an antibody specific to VDR (Fig. 3). At both time points, we observed an increase in VDR protein after combination of 1,25-D$_3$ with DEX above that seen with 1,25-D$_3$ alone. A second band of reduced mobility is visible in all samples treated with 1,25-D$_3$. The appearance of this band is consistent with previous studies that demonstrated phosphorylation of the murine VDR upon the addition of ligand (45). Although the function of this phosphorylation is unclear, it may be important for VDR activation (46).

Because VDR typically functions as a heterodimer with RXR, we examined the effects of 1,25-D$_3$ alone or in combination with DEX, on RXR protein levels at 4 h and 48 h after treatment of SCC cells. There are three known isoforms of RXR, α, β, and γ (47), and we examined the expression of all three potential partners. Treatment with 1,25-D$_3$ increased RXRα protein levels, whereas treatment with the combination of 1,25-D$_3$ and DEX resulted in RXRα levels that were comparable with the level in untreated cells (Fig. 3). RXR β protein levels were relatively constant in all treatment groups, and RXRγ was below the level of detection in SCC cells (data not shown).

1,25-D$_3$ Treatment Increases GR Protein Levels and Ligand Binding. To test whether 1,25-D$_3$ could affect DEX signaling through the GR, we examined GR ligand binding and protein levels in SCC cells treated with 1,25-D$_3$ and/or DEX. To examine total cellular DEX binding, we used a single-point saturation assay to determine maximum DEX-binding capacity. Treatment with 1,25-D$_3$ was found to significantly increase DEX-binding capacity to 59.7 ± 5.3 fmol/mg cellular protein compared with 15.0 ± 1.5 fmol/mg for untreated cells (P < 0.01, Student’s t test). Thus, as was seen with the VDR, there is cross-regulation of GR binding by 1,25-D$_3$. 1,25-D$_3$ treatment also increased GR protein levels (Fig. 3). However, unlike the regulation of VDR, which is increased further by the combination of 1,25-D$_3$ and DEX, this combination caused a decrease in GR protein levels compared with control or 1,25-D$_3$ treatment alone and similar to that seen with DEX alone (Fig. 3).

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**Table 1** DEX enhances the cell cycle arrest caused by 1,25-D$_3$. SCC cells were treated for 24 h with either ethanol vehicle control, 1,25-D$_3$ (10 nM) ± DEX (500 nM) ± RU486 (2.5 µM). Nuclei were then stained with propidium iodide and analyzed for DNA content by flow cytometry. The results shown are the mean ± SE for 2–5 separate experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G$_0$/G$_1$</th>
<th>S</th>
<th>G$_2$/M</th>
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<tr>
<td>Ethanol</td>
<td>49.4 ± 2.6</td>
<td>41.0 ± 2.3</td>
<td>9.2 ± 1.3</td>
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<td>1,25-D$_3$</td>
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<td>17.4 ± 3.3</td>
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<tr>
<td>DEX</td>
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</tr>
<tr>
<td>RU486</td>
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<td>8.5 ± 0.4</td>
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<tr>
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<td>11.3 ± 2.5</td>
<td>6.9 ± 0.5</td>
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<td>10.0 ± 2.0</td>
</tr>
<tr>
<td>1,25-D$_3$ + DEX + RU486</td>
<td>72.5 ± 4.2</td>
<td>19.4 ± 3.8</td>
<td>8.4 ± 0.6</td>
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functional assays are required to more accurately assess the effect of 1,25-D₃ on GR activity.

**DEX Does Not Increase 1,25-D₃-mediated Transcriptional Activity and 1,25-D₃ Does Not Increase DEX-mediated Transcriptional Activity.** Because 1,25-D₃ and DEX both affected the protein levels and ligand binding of the other ligand’s receptor, we tested the ability these agents to affect the transcriptional activation of VDRE and GRE reporters. We transiently transfected SCC cells with a VDRE or GRE reporter construct and assayed CAT activity after treatment with 1,25-D₃ and/or DEX. Whereas 1,25-D₃ treatment resulted in a dramatically increased level of CAT activity in cells transfected with the VDRE as expected, the combination of 1,25-D₃ and DEX was consistently lower (three independent experiments; 77.1 ± 24.4%) than after treatment with 1,25-D₃ alone, although this trend did not reach statistical significance (Fig. 4A). Thus, although the addition of DEX to 1,25-D₃ treatment caused an increase in VDR protein levels and ligand binding, it did not enhance the ability of 1,25-D₃ to activate transcription of a VDRE-reporter construct. This result correlates with the effects on RXR that were observed for the combination, indicating that RXR may be limiting (Fig. 3). GRE reporter activity was increased by DEX as expected, but the addition of 1,25-D₃ did not affect this reporter (Fig. 4B). Thus, whereas treatment with 1,25-D₃ induces transcriptional activation at a VDRE, and DEX induces transcriptional activation at a GRE, the level of activity of these reporters is not increased by the combination of 1,25-D₃ and DEX more than the appropriate ligand alone.

**Reduction of Phospho-Erk1/2 and Phospho-Akt by 1,25-D₃ and DEX.** We have recently reported that 1,25-D₃ can reduce the levels of phospho-Erk1/2 and phospho-Akt (34). In this study, we wanted to determine whether DEX enhanced the effects on these two signaling pathways that are critical regulators of proliferation and survival. To test this possibility, we performed immunoblots on SCC cells treated with the combination of 1,25-D₃ and DEX or either agent alone using antibodies that recognize only the phosphorylated, active form of Erk1/2 and Akt. The levels of the phosphorylated, active Erk1/2 were reduced in cells treated with 1,25-D₃ and another reduction was observed with the addition of DEX (Fig. 5). Similarly, a reduction of phospho-Akt was observed with 1,25-D₃ and an additional decrease was seen with the combination of 1,25-D₃ and DEX. The GR antagonist, RU486, inhibited the effects of DEX on both Erk1/2 and Akt, suggesting that the GR is required for these effects.

To test whether the decrease in phospho-Erk1/2 and phospho-Akt could also be observed in vivo, we treated mice bearing SCC tumors with 0.25 g/mouse for 3 days of 1,25-D₃ or saline control. Animals treated on these schedules demonstrated an enhanced
antitumor activity for the combination compared with either agent alone (Ref. 18, and data not shown), and this schedule is being used for current clinical trials. Tumors were harvested 6 h after the final treatment, and tumor lysates were prepared. Western blot analyses of three animals/group revealed that average levels of phospho-Erk1/2 and phospho-Akt were decreased by 1,25-D3 and reduced further by the combination of 1,25-D3 and DEX (Fig. 6).

**DISCUSSION**

Despite evidence of the clinical utility of the combination of 1,25-D3 and DEX in cancer therapy, the mechanism(s) by which this combination acts to inhibit proliferation has not been extensively studied. A better understanding of the molecular consequences of this therapy may lead to the identification of predictors and markers of response, as well as potential new targets for improved therapies. In this report, we examined whether DEX enhances the cell cycle arrest or apoptotic effects of 1,25-D3 or both. We also examined the possibility that nuclear receptor cross-talk may play an important role in the antiproliferative effects of this combination. Finally, we examined some of the signaling pathways that are known to be affected by these agents when given alone.

Assessment of the cell cycle and apoptotic effects of the combination of 1,25-D3 and DEX may indicate whether this therapy is cytostatic or may also be cytotoxic. Such information is useful when additional agents are incorporated into new therapeutic regimens involving 1,25-D3 and DEX. In this report, we demonstrate that DEX enhanced the ability of 1,25-D3 to induce both G0/G1 arrest and apoptosis. Thus, addition of other agents to this combination that act through either of these processes may be beneficial. The modest toxicity and oral availability of 1,25-D3 and DEX makes such multidrug regimens attractive. Furthermore, the ability of RU486 to inhibit the antiproliferative, cell cycle, and apoptotic effects of DEX in this combination indicates that the GR is likely required for the enhancement of 1,25-D3 activity by DEX. Thus, agents that affect the GR would also be expected to have consequences on this combination.

Because DEX was found to enhance both the cell cycle and apoptotic effects of 1,25-D3, we hypothesized that DEX may be acting via cross-talk with the VDR, resulting in an increase in the genomic effects of 1,25-D3. Previous studies demonstrating an increase in 1,25-D3 binding in SCC cells treated with DEX (18) as well as numerous examples of cross-talk between nuclear receptors (24, 26, 27) supported this possibility. If such an interaction occurs, the increased expression of vitamin D-responsive genes, possibly including transforming growth factor β (48), insulin-like growth factor binding protein 3 (49), insulin-like growth factor binding protein 5 (50), and others (51), could potentially explain the enhanced antitumor activity of the combination. We show here that DEX increased VDR protein levels more than either agent alone. This finding is supported by preliminary clinical data that shows a similar trend of increased VDR in the peripheral blood monocytes of patients treated with 1,25-D3 and DEX than after treatment with 1,25-D3 alone (data not shown). However, reporter activity from a construct containing two copies of a VDRE was not significantly different after treatment with the combination in comparison to 1,25-D3 alone. Thus, the enhancement of 1,25-D3’s antiproliferative

Fig. 5 Effects of 1,25-D3 and DEX on phospho-Erk1/2 and phospho-Akt levels _in vitro_. Western blots for phospho-Erk1/2 and phospho-Akt are shown for whole cell lysates of SCC cells treated with 1,25-D3 (10 nM) and/or DEX (500 nM) ± RU486 (2.5 μM) for 48 h. A control blot for actin is also shown to demonstrate accurate quantitation and equal loading. Densitometric values for the combined density of phospho-Erk1 and phospho-Erk2 (p42 and p44) and for phospho-Akt were normalized to actin and the vehicle control and are shown below each lane. These results are representative of three independent experiments.

Fig. 6 Effects of 1,25-D3 and DEX on phospho-Erk1/2 and phospho-Akt levels _in vivo_. A, Western blots for phospho-Erk1/2 and phospho-Akt are shown for lysates of SCC tumors harvested 6 h after the final treatment with 1,25-D3 (0.25 g/mouse for 3 days) and/or DEX (9 μg/mouse for 4 days). Three animals/group were used. B, densitometric quantitation of the values for the three animals in each group normalized to actin and the saline control group are shown (±SD).
effects by DEX is not likely to be the result of a generalized increase in transcriptional activation by VDR.

Still, we are not yet able to exclude certain other potential mechanisms for nuclear receptor cross-talk. For example, the reporter construct that was used included only DR3-type VDRE, which contains two direct repeats of six nucleotides spaced by three bases. Although the DR3-type represents the majority of known VDRE, multiple other VDRE types exist and may be regulated differently (24, 52). Interestingly, Carlberg et al. (53) have demonstrated that a vitamin D analogue that preferentially activates I(9) type (inverted palindrome spaced by nine nucleotides) elements compared with DR3-type elements has strong antiproliferative effects, yet reduced calcemic effects. By analogy, it remains possible that DEX alters the specificity of the VDR for certain VDRE types. In addition, the fact that both VDR and GR share a large number of coactivators and corepressors makes the possibility of preferential transactivation from certain VDRE more plausible, because shared components required for certain functions may become limiting. The increased number of response elements in the setting of a reporter assay may reduce further the availability of co-activators. Finally, there are likely multiple genes that contain both VDRE and GRE in their promoters. For example, the promoter region of the human endoglin (CD105) gene contains both a VDRE and a GRE (54). Furthermore, regulation of the human osteocalcin gene by both 1,25-D$_3$ and DEX has been shown to occur through distinct sequences in the osteocalcin gene promoter region (55). Additional studies will be necessary to obtain a more complete understanding of the effects of DEX on a variety of different VDRE and GRE. In addition, as the relevant target genes become known it will be possible to examine the relevant promoters.

Evidence also exists that there is a membrane-bound VDR (56, 57), as well as secondary GRs (58). These receptors could also be involved in the antiproliferative activity of this combination. However, preliminary data obtained using 1$(\beta$-25-D$_3$), an antagonist of the membrane VDR, suggests that this receptor does not play a role in 1,25-D$_3$-induced alterations in the cell cycle distribution of SCC cells (data not shown).

If DEX is able to enhance signaling through the VDR, then the combination of 1,25-D$_3$ and DEX should affect the same signaling pathways as 1,25-D$_3$, only to a greater degree. Even if receptor cross-talk is not critical to the enhanced antiproliferative effects of this combination, understanding the effects of this combination on growth and survival pathways should prove useful for developing new therapies. Thus, we tested whether DEX enhances some of the key molecular changes that are observed after treatment with 1,25-D$_3$.

We have shown previously that 1,25-D$_3$ causes a reduction of phospho-Erk1/2 levels, particularly in the population of cells that were floating after treatment (34). This effect may be important, as Erk is central to a critical signaling pathway that regulates proliferation. The activity of this pathway is frequently increased in cancer because of mutations in Ras or other components of the pathway, which can make tumor cells resistant to normal growth controls (35). Here, we found that the levels of the phosphorylated, active form of Erk1/2 were reduced more by the combination of 1,25-D$_3$ and DEX than by either agent alone. This effect occurred both in vitro (Fig. 5) and in vivo (Fig. 6).

Thus, reduction of phospho-Erk1/2 is a potentially useful marker of response to this therapy. In addition, the effects on Erk may also explain the reduction of p21$^{CIP-1/WAF1}$ that is seen with these treatments (41), because active Erk can induce p21$^{CIP-1/WAF1}$ expression (35).

Reduction of the activity of the Erk pathway may also sensitize cells to chemotherapeutic agents (59). In fact, we have reported that 1,25-D$_3$ does sensitize SCC cells to platinum and taxanes (33, 60). Thus, the greater reduction of phospho-Erk1/2 that is caused by the combination of 1,25-D$_3$ and DEX may sensitize cells further to chemotherapeutic agents, a possibility that we are currently investigating.

The mechanism of the reduction of phospho-Erk1/2 may be attributable to our recent finding that 1,25-D$_3$ induces cleavage of mitogen-activated protein kinase/Erk kinase (MEK), an upstream activator kinase of Erk (34). It is possible that DEX enhances this effect. Another possibility is that DEX functions independently, as demonstrated by Croxtall, et al. (38). They showed that DEX reduces the level of phospho-Erk1/2 by a GR-dependent, but transcription-independent, mechanism in A549 cells. Furthermore, they showed that the inhibition occurred at the level of Grb2 association with phosphorylated epidermal growth factor receptor.

Similar to the results with phospho-Erk1/2, we reported previously that 1,25-D$_3$ reduces the level of phospho-Akt, particularly in the detached cells (34). The Akt signaling pathway is also critical for cell survival, and it is commonly over-expressed in cancer cells with mutations of PTEN, a phosphatase that can reduce Akt activity, or other members of this signaling pathway (36). Increased Akt activity also correlates with prostate cancer progression and a reduction in p27$^{Kip1}$ expression (61). Furthermore, Hisatke et al. (62) recently demonstrated that a vitamin D analogue induced the expression of PTEN and p27$^{Kip1}$ in HL-60 cells as well as causing G$_2$/M cell cycle arrest. Inhibitors of PI3K, which lead to a reduction of phospho-Akt levels, have also been shown to induce p27$^{Kip1}$ and to decrease p21$^{CIP-1/WAF1}$ levels (63), similar to the effects of 1,25-D$_3$ in SCC (41). Thus, reductions in Akt activity may be critical for the cell cycle arrest induced by 1,25-D$_3$ via induction of p27$^{Kip1}$. In addition, decreased Akt activity would likely lead to lower phosphorylation levels of Bad, a proapoptotic member of the Bcl-2 super-family, which is inactivated upon phosphorylation by Akt (36); thereby potentially sensitizing cells to chemotherapeutic agents such as paclitaxel (64). Indeed, we have shown 1,25-D$_3$ and paclitaxel act synergistically in the SCC model (60).

In this study, we demonstrate a reduction of phospho-Akt levels in SCC cells treated with 1,25-D$_3$ and DEX that is greater than the effects of either agent alone. This modulation of phospho-Akt levels was observed both in vitro and in vivo. The mechanism for this additional reduction is uncertain, but may be caused by an inhibition of epidermal growth factor receptor and/or insulin-like growth factor receptor signaling, as has been demonstrated for DEX in other cell types (38). For example, Singleton et al. (37) demonstrated that DEX can inhibit the up-regulation of phospho-Akt induced by IGF-1 in myoblasts. This effect correlated with an increased expression of the p85a subunit of PI3K, which is a negative regulator of PI3K activity and therefore reduces Akt activity.
As in the cell cycle and PARP experiments, RU486 inhibited the effects of DEX on phospho-Erk1/2 and phospho-Akt, making the combination seem similar to 1,25-D3 alone. These results suggest that binding of DEX to the GR is critical for the enhancement of the antiproliferative effects of 1,25-D3 by DEX. However, this observation is not sufficient to conclude that transcriptional activation by GR is necessary, because RU486 can also block some “nongenomic” effects of DEX (65).

Regardless of the mechanism by which DEX affects Erk1/2 and Akt in the presence of 1,25-D3, the phosphorylation status of these two molecules is potentially useful as a marker of the antiproliferative response to this combination therapy. In fact, preliminary data indicate that a decrease in phospho-Erk1/2 is observed in peripheral blood monocytes of patients treated with 1,25-D3 and DEX that is greater than the decrease that is observed after treatment with 1,25-D3 alone (data not shown). However, it is still uncertain whether these cells reflect the situation in the patient’s tumor. Attempts to validate these markers in tumor samples are currently underway. In addition, the importance of these signaling molecules must be tested operationally.

In conclusion, we demonstrated that the combination of 1,25-D3 and DEX affects both the Erk and Akt signaling pathways and leads to greater cell cycle arrest and apoptosis than either agent alone. Furthermore, we demonstrated that the mechanism of the enhanced antiproliferative effects of this combination is more complex than a generalized increase of transcriptional activation by VDR. Finally, the ability of DEX to decrease the hypercalcemia toxicity should allow higher doses of 1,25-D3 to be given safely, thereby potentially reaching concentrations at which these agents act effectively and synergistically.

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Combination of 1α,25-Dihydroxyvitamin D$_3$ with Dexamethasone Enhances Cell Cycle Arrest and Apoptosis: Role of Nuclear Receptor Cross-Talk and Erk/Akt Signaling

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