1,25-Dihydroxycholecalciferol (1,25-D₃) Inhibits the Growth of Squamous Cell Carcinoma and Down-Modulates p21Waf1/Cip1 in Vitro and in Vivo


ABSTRACT

1,25-Dihydroxycholecalciferol (1,25-D₃) has significant antitumor effects in the murine squamous cell carcinoma (SCC) tumor model in vitro and in vivo. We investigated the basis for this antiproliferative activity and found that, in vitro, 1,25-D₃ administration is associated with altered expression of cell cycle regulatory proteins, treatment results in retinoblastoma dephosphorylation, decreased expression of p21Waf1/Cip1 (p21) mRNA and protein, and increased expression of p27kip1 (p27) mRNA and protein. Dexamethasone, which acts synergistically with 1,25-D₃ to inhibit SCC proliferation, enhanced 1,25-D₃-induced down-modulation of p21 without affecting the ability of 1,25-D₃ to increase p27 expression. 1,25-D₃ did not induce cleavage of poly(ADP-ribose) polymerase. These in vitro data suggest that 1,25-D₃ exerts antitumor activity in SCC by perturbing cell cycle progression rather than by inducing apoptosis. In vivo, a 1,25-D₃ treatment regimen that results in a decrease in SCC tumor volume is associated with a statistically significant decrease in intratumoral p21 expression. p21 expression is not changed in tumors isolated from control animals or animals treated with a nontherapeutic dose of 1,25-D₃. Intra-tumoral p27 levels were not modulated by 1,25-D₃ treatment. Thus, both in vitro and in vivo, 1,25-D₃-mediated growth inhibition is associated with p21 down-modulation.

INTRODUCTION

Considerable evidence suggests that 1,25-D₃, the active metabolite of vitamin D, may play an important role in human cancer. Reduced serum concentration of 1,25-D₃ is associated with an increased risk of breast, prostate, and colon cancer (1). Increased serum levels of 1,25-D₃ binding protein and polymorphisms in the VDR are also associated with an increased risk of prostate cancer (2). In addition, high level VDR expression correlates with a favorable prognosis in colorectal cancer (3).

In vitro, 1,25-D₃ promotes the differentiation of myeloid leukemias (4–6) and inhibits the proliferation of prostatic (7), breast (8, 9), colon (10), and pancreatic carcinomas (11). In vivo, 1,25-D₃ inhibits tumor cell growth in breast (9), prostate (7), colon (12), and squamous cell (13) animal tumor model systems. The antiproliferative activity of 1,25-D₃ in these different systems has been linked to its ability to promote cellular differentiation, cell cycle arrest, and apoptosis (14).

In prostate carcinoma cell lines, the antiproliferative effects of 1,25-D₃ require VDR expression (15, 16). 1,25-D₃ binding activates the VDR, allowing it to modulate the transcription of target genes with promoters containing a vitamin D response element (17).

We have demonstrated that 1,25-D₃ has significant antitumor activity in vitro and in vivo in the murine SCC model (13). 1,25-D₃ induces SCC to undergo growth arrest with a significant percentage of cells in G0-G1 after 24 h of treatment in vitro (18). We have also shown that 1,25-D₃ activity in SCC is enhanced by concomitant treatment with dex (19). In SCC tumor-bearing mice, dex increases VDR ligand binding within tumor and kidney, but not in intestine, which may explain its ability to enhance 1,25-D₃ antitumor activity while blocking 1,25-D₃-induced hypercalcemia (19).

In hematopoietic cells (5, 20) and epithelial (11, 21, 22) cell lines, 1,25-D₃-mediated cell cycle arrest correlates with increased expression of the cdk inhibitors p21Waf1/Cip1 or p27kip1. These proteins negatively regulate cell cycle progression by binding to and inhibiting cyclin:cdk complexes (23). Agents promoting G0-G1 arrest and differentiation in HL60 cells, such as 1,25-D₃, increase p21 (6, 24) and p27 (20) expression. 1,25-D₃-mediated cell cycle arrest in these cells is associated with decreased cdk2 and cdk6 activity (25). In U937 cells, 1,25-D₃ induces p21 and p27 expression, and this increase is associated with the presence of a vitamin D response element in the p21 promoter (5). 1,25-D₃ antiproliferative activity in pancreatic cancer lines in vitro has also been attributed to increased p21 and p27 expression (11). These studies describe modulation of p21 and p27 by 1,25-D₃ in tissue culture systems, although comparable in vivo studies have not yet been reported. To investigate the basis for 1,25-D₃ antiproliferative activity in SCC, studies were undertaken to examine whether p21 or p27 were modulated in vitro and in vivo by 1,25-D₃ and whether these effects correlated with tumor growth inhibition.

MATERIALS AND METHODS

Chemicals and Reagents. Vitamin D₃, 1,25-D₃ (Hoffmann-LaRoche, Nutley, NJ) was recombinantly produced in 100% ethanol and stored protected from light under a layer of nitrogen gas at -70°C. All handling of 1,25-D₃ was performed with indirect lighting. Dexamethasone (dexamethasone; Sigma Chemical Co., St. Louis, MO) was dissolved in dH₂O to 25 mg/ml. Dilutions of 1,25-D₃ or dex were made in tissue culture media just before use. The antibodies used were: polyclonal rabbit anti-VDR (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti-Rb (Clone G3–245; Pharmingen, San Diego, CA), monoclonal mouse anti-PARP (Enzyme Systems, Livermore, CA), polyclonal rabbit anti-p21 (PharMingen), and polyclonal rabbit anti-p27 (PharMingen). Anti-rabbit and antimouse horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Life Sciences (Arlington Heights, IL) and Promega (Madison, WI), respectively.

Tumor Cells and Model System. For these studies, the murine SCC-VII/SF tumor model was used. SCCVII/SF is a moderately well differentiated SCC derived from a spontaneously arising tumor of the C3H mouse (26). SCCVII/SF cells were maintained in female C3H/HeJ mice, 6–10 weeks of age, obtained from The Jackson Laboratory (Bar Harbor, ME), as described previously (13). The mice were age- and sex-matched for experimental use. Animals were used in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. For in vitro studies, SCC cells were grown in RPMI 1640 plus 15% FCS and only passed twice. In vitro studies were initiated by treating subconfluent SCC cultures with 5.0 ml of fresh media containing either 0.004% ethanol vehicle, 10 nm 1,25-D₃, 500 nm dex, or 10 nm 1,25-D₃ + 500 nm dex/T25 flask.

Preparation of Cell Lysates. Protein was extracted from in vitro-treated cells and from tumors harvested from treated animals using lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris (pH 8.0), 150 mM NaCl, 0.6 mM PMSF, 1 mM EDTA, protease and phosphatase inhibitors] and a TissueLyser (Sigma-Aldrich). Protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Samples were stored at -80°C until used.

Received 12/1/98; accepted 4/2/99.

The cost of publication of this article was defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Funded by NIH/National Cancer Institute Grant CA67267 (to C. S. J.).

2 To whom requests for reprints should be addressed, at University of Pittsburgh, Department of Pharmacology, W1002 Biomedical Science Tower, Pittsburgh, PA 15213. Phone: (412) 648-2344; Fax: (412) 648-9856; E-mail: pah13+@pitt.edu.

3 The abbreviations used are: 1,25-D₃, 1,25-dihydroxycholecalciferol; dex, dexamethasone; SCC, squamous cell carcinoma; PARP, poly(ADP-ribose) polymerase; VDR, vitamin D receptor; cdk, cyclin-dependent kinase; Rb, retinoblastoma.
and 5 μg/ml leupeptin]. To extract proteins from in vitro cultures, monolayers were washed twice with PBS, and 200 μl of lysis buffer were added/T25 flask. Flasks were rocked for 30 min at 4°C. Lysates were transferred to 1.5-ml Eppendorf tubes and clarified by centrifugation at 13,000 rpm for 10 min at 4°C.

Lysates were also prepared from tumors that were flash frozen in liquid nitrogen immediately at the time of harvest. Frozen tumors were pulverized, then homogenized for 45 s at 4°C in 1.5–3.0 ml of lysis buffer. Homogenates were stored on ice for 30 min and then clarified, as described above. Clarified protein lysates were transferred to fresh tubes and stored at -80°C until use. Protein concentrations were determined in duplicate using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), according to manufacturer’s directions.

Western Blot Analysis. Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions, and then electrophoretically transferred to poly(vinylidene difluoride) membranes (NEN Life Science Products, Boston, MA) overnight at 4°C. At room temperature, membranes were blocked for a minimum of 1 h in a 5% w/v solution of nonfat milk in TBST [10 mM Tris (pH 7.6), 150 mM NaCl, and 0.05% Tween 20], then incubated for 1 h with primary antibody. The blots were washed three times in TBST and subsequently incubated with secondary antibody conjugated with horseradish peroxidase for 1 h. The blots were again washed, and the proteins were detected using Renaissance Western blot chemiluminescence reagents (NEN Life Science Products).

Northern Blot Analysis. Total RNA was extracted from in vitro-treated cells using the Ultraspec RNA isolation system (Biotecx Laboratories, Houston, TX), according to manufacturer’s directions. RNA was electrophoresed through formaldehyde-agarose gels and transferred to GeneScreen (NEN Life Science Products) overnight at room temperature.

For p21 Northern blots, the EcoRI fragment of the murine p21 cDNA (generously provided by Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD) was labeled with [32P]-dCTP using the random primed DNA labeling kit from Boehringer Mannheim (Indianapolis, IN). After prehybridization, labeled probe was diluted and incubated with the membrane overnight at 43°C. The blots were washed under standard conditions and exposed to film at -80°C.

For p27 Northern blots, the HindIII-EcoRI fragment of murine p27 was isolated from the plasmid AE1xox(+)mp27 (generously provided by Dr. J. Massagué, Memorial Sloan-Kettering Cancer Center, New York, NY). The gel-purified fragment was labeled using the random primer fluorescein labeling kit from NEN Life Sciences Products. The cDNA labeling reaction and subsequent Northern blots were performed according to the manufacturer’s instructions.

Densitometry. Densitometry of autoradiographs was performed using a Molecular Dynamics personal densitometer equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

We previously demonstrated that 1,25-D3 induces murine SCC cells to undergo G0-G1 arrest (18). To examine the kinetics with which cell cycle changes occurred in response to 1,25-D3 treatment, cell lysates were prepared after in vitro treatment with ethanol vehicle or 1,25-D3. Rb phosphorylation was examined by Western blot using an antibody that detects both hyperphosphorylated and hypophosphorylated Rb. Whereas hyperphosphorylated Rb promotes cell cycle progression, hypophosphorylated Rb binds E2F and blocks exit from G1 progression, whereas the faster migrating species (pRb) results from Rb dephosphorylation. Only the hypophosphorylated form of Rb was detected in cells treated with 1,25-D3 for 24 h, consistent with the observation that 1,25-D3 induces G0-G1 arrest in SCC (Fig. 1).

In several cell types, 1,25-D3-mediated cell cycle arrest has been associated with increased expression of the cdk inhibitors p21 and p27 (5, 11, 21). To determine whether 1,25-D3-mediated cell cycle arrest in SCC is associated with altered expression of these molecules and whether dex affects the regulation of p21 and/or p27 by 1,25-D3, cell lysates were prepared after in vitro treatment with either ethanol, dex, 1,25-D3, or 1,25-D3 + dex and analyzed by Western blot. At 4 h after treatment, the expression of p21 and p27 varied <20% between control and experimental groups (Fig. 2, A and B). However, at 24 h, treatment with either 1,25-D3 or dex reduced p21 expression 2- to 3-fold compared with ethanol, and p21 was reduced 10-fold after treatment with 1,25-D3 + dex. Although dex had no effect on p27 expression, 1,25-D3 treatment resulted in an ~2-fold increase in p27 expression at 24 h, which was not further enhanced by concomitant dex exposure (Fig. 2B).

Northern analyses were conducted to determine whether 1,25-D3-mediated changes in p21/p27 were associated with altered transcript expression. A 2-h treatment with 1,25-D3, dex, or 1,25-D3 + dex resulted in a 2- to 3-fold increase in p21 mRNA levels compared with control, whereas treatment for 24 h resulted in a 2-fold decrease in the steady-state level of p21 mRNA (Fig. 3A). At the 2-h time point, p27 transcripts were expressed at a comparable level in all treatment groups. By 24 h, p27 transcripts increased nearly 2-fold after treatment with either 1,25-D3 or dex and increased ~4-fold after 1,25-D3 + dex treatment (Fig. 3B). Thus, alterations in p21 and p27 transcript levels may contribute to the changes in p21 and p27 protein levels observed after 24 h of 1,25-D3 treatment.

1,25-D3 has been shown to induce apoptosis in MCF-7 cells (28, 29). Therefore, the antiproliferative activity of 1,25-D3 in SCC could also result from the induction of apoptosis. To investigate whether 1,25-D3 induces SCC apoptosis, cell lysates were prepared after in vitro treatment with either ethanol, dex, 1,25-D3, or 1,25-D3 + dex and analyzed by Western blot for PARP cleavage. PARP is cleaved into a characteristic 85-kDa fragment in cells undergoing apoptosis (30). 1,25-D3 induces SCC apoptosis, cell lysates were prepared after in vitro treatment with either ethanol, dex, 1,25-D3, or 1,25-D3 + dex and analyzed by Western blot for PARP cleavage. PARP cleavage, although SCC cells express this marker of apoptosis at a low level, the level of PARP cleavage is not modulated significantly by 1,25-D3, dex, or the combination. The inability of 1,25-D3 to induce apoptosis in SCC was confirmed in flow cytometric studies, in which only a low level of annexin binding activity was detected in control cultures and cultures treated with 1,25-D3 (data not shown).

These results suggest that 1,25-D3 exerts antiproliferative activity in SCC in vitro by perturbing cell cycle rather than by inducing apoptosis. To determine whether p21 and p27 are also modulated by 1,25-D3 in vivo, SCC tumor-bearing animals were treated with either: (a) saline control; (b) 0.75 μg of 1,25-D3 daily, for 3 days

Fig. 1. 1,25-D3 modulates Rb phosphorylation status in SCC cells. Whole cell lysates were prepared from subconfluent SCC treated in vitro with either ethanol or 10 nM 1,25-D3 for 4–24 h. Protein (30 μg) from each lysate was analyzed by Western blot using an anti-Rb antibody. The bands designated pRb and ppRb correspond to hyperphosphorylated Rb and hypophosphorylated Rb, respectively. The results shown are representative of two independent experiments.

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Different treatment regimens were selected to determine whether 1,25-D$_3$-mediated effects on cdk inhibitor expression correlated with significant inhibition of tumor growth. Three days of 1,25-D$_3$ treatment has been shown to be effective at inducing a significant decrease in SCC tumor size (19). As shown in Table 1, SCC tumor volumes increased ~3-fold over a 96-h period in control, saline-injected animals. Tumors from animals treated with a single dose of 1,25-D$_3$ showed only a 1.5-fold increase in volume over the same time course, indicating that a single injection of 1,25-D$_3$ was sufficient to slow tumor growth significantly. However, none of the tumors from animals treated with the single dose therapy shrunk to below pretreatment volume. In contrast, a decrease in tumor volume was observed in 8 of 10 animals receiving a daily dose of 1,25-D$_3$ for 3 days, resulting in a statistically significant decrease in fractional tumor volume at 96 h.

At 4, 8, and 24 h after the final injection of 1,25-D$_3$, the SCC tumors were harvested. Lysates were prepared from the tumors and then analyzed by Western blot for expression of p21, p27, and VDR. VDR was used as a positive control in these experiments because it accumulates in response to ligand binding (31), allowing us to verify that 1,25-D$_3$ was delivered to the tumor. As shown in Table 2, repeated administration of 1,25-D$_3$ in vivo was associated with a statistically significant decrease in p21 expression within tumors. Relative to saline-injected controls, p21 expression was decreased at 4, 8, and 24 h in tumors harvested from animals receiving three injections of 1,25-D$_3$; p21 expression was not similarly down-modulated at 4 h or at 8 h after treatment in tumors harvested from animals receiving a single injection of 1,25-D$_3$; p21 levels did decline slightly in these samples after 24 h of treatment. Unlike p21, p27 expression was comparable in tumors harvested from control animals and animals treated with 1,25-D$_3$ at each of the time points examined. Thus, a 1,25-D$_3$ treatment that resulted in SCC tumor regression was associated with a decrease in p21 expression, but no change in p27 expression. In animals treated with either one or multiple doses of 1,25-D$_3$, tumor cell expression of VDR was elevated 4 h and 8 h after treatment and declined to control levels within 24 h. This modulation of VDR expression confirms delivery of 1,25-D$_3$ to the tumors.

DISCUSSION

We have shown that 1,25-D$_3$ inhibits proliferation of the murine SCC SCCVII/SF in vitro and in vivo (13, 18, 19). 1,25-D$_3$-treated SCC cells accumulate in G$_0$-G$_1$ (18) and, after 24 h of in vitro treatment, display a decrease in Rb phosphorylation (Fig. 1). These data suggest that the in vitro antitumor activity of 1,25-D$_3$ in SCC results from inhibition of cell cycle progression. Loss of Rb phosphorylation in response to 1,25-D$_3$ treatment is associated with an ~2-fold increase in expression of the cdk inhibitor p27 and a nearly 3-fold decrease in expression of the related cdk inhibitor p21. In vivo, administration of 1,25-D$_3$ (0.75 μg/day for 3 days) to SCC tumor-bearing mice resulted in a reduction in tumor volume that was associated with a statistically significant decrease in p21 expression within tumors. However, intratumoral p27 levels were not modulated by 1,25-D$_3$ treatment. These experiments, which represent the first investigation of in vivo modulation of cdk inhibitors in response to
1,25-D$_3$ administration, suggest that the growth inhibitory effects of 1,25-D$_3$ in SCC either require or result in p21 down-modulation.

In vitro, evidence of 1,25-D$_3$-mediated G$_0$-G$_1$ arrest in SCC is observed as early as 24 h after treatment (Fig. 1 and Ref. 18). At this time point, p27 expression is increased and p21 expression is decreased within 1,25-D$_3$-treated cells (Fig. 2, A and B). Thus, changes in one or both of these molecules may be important for the in vitro antitumor activity of 1,25-D$_3$. Dex, which acts synergistically with 1,25-D$_3$ to inhibit SCC proliferation in vitro and in vivo (19), enhances 1,25-D$_3$-induced down-modulation of p21 without affecting the ability of 1,25-D$_3$ to up-modulate p27 (Fig. 2, A and B). These data may indicate that the changes in p21 are most critical to the antitumor activity of 1,25-D$_3$. This hypothesis is further supported by our finding that 1,25-D$_3$ mediates p21 reduction in vivo without modulating p27.

p21 promotes cell cycle arrest in G$_0$G$_1$ by binding to G1 cyclin:cdk complexes and inhibiting their activity, which is required for entry into S phase (23). Enforced overexpression of p21 in tumor cells using an adenoviral vector (32) or a tetracycline-inducible system (33) results in tumor growth inhibition in vitro and in vivo, indicating that p21 possesses tumor suppressor activity. Moreover, p21 induction by 1,25-D$_3$ correlates with growth inhibition in pancreatic (11) and prostate cancer cell lines (21). It was, therefore, initially surprising that the antiproliferative activity of 1,25-D$_3$ in SCC is associated with p21 reduction rather than p21 induction. In contrast to these earlier studies, our findings suggest that p21 has growth and/or survival-promoting activities in SCC that can be overcome by 1,25-D$_3$ administration; such unique roles for p21 have been described recently (34, 35).

How might reduced p21 levels lead to SCC growth inhibition? We previously reported that 1,25-D$_3$ caused prominent morphological changes in SCC in vitro (increased cell size and cytoplasmic spreading), consistent with cellular differentiation (13). Similar changes in cell morphology and p21 expression were observed in vitro in human SCC cells induced to differentiate by stable RXR$\gamma$ expression (36). Inhibition of p21 expression was also recently shown to be necessary for keratinocytes to complete terminal differentiation when cultured in low calcium medium (37). These results support the hypothesis that the in vitro antiproliferative activity of 1,25-D$_3$ results from its ability
to induce SCC differentiation, which may require p21 reduction for its completion.

Loss of p21 might also lead to SCC growth inhibition by triggering an apoptotic cascade. In endothelial cells deprived of growth factors, proteolytic cleavage of p21 results in inappropriate cdk2 activation and apoptosis (38). Although these data suggest that loss of p21 expression itself can act as a trigger for cell death, we find no evidence that 1,25-D3 induces SCC apoptosis in vitro, at a time when p21 expression is reduced. It is possible that 1,25-D3-treated SCC cells do not undergo apoptosis in response to p21 down-modulation in vitro due to a compensating increase in p27 expression, which itself has been shown to protect certain cells against apoptosis (39). However, a different situation may exist in vivo, where 1,25-D3 decreases p21 expression without increasing p27 (Table 2). In this case, loss of p21 may result in cdk2 activation and apoptosis. Consistent with such a hypothesis, we find that 1,25-D3 induces a potent apoptotic response in MLL cells, in which both p21 and p27 are down-modulated in response to treatment. Initiation of an apoptotic response in vivo would account for the reduction in tumor volume that is observed after three daily injections of 1,25-D3.

p21 has been reported to protect cells from apoptosis, and loss of p21 has been shown to sensitize cells to both DNA- (40) and microtubule-damaging agents (34). In a human xenograft model, HT116 tumors harboring homozygous deletion of p21 regressed after radiation therapy while parental HT116 tumors progressed, further supporting the notion that p21 must be eliminated before cells become susceptible to apoptosis (40). HT116 cells deficient in p21 expression also displayed increased susceptibility to cisplatin and nitrogen mustard in vivo (41). We have previously demonstrated that SCC cells are rendered significantly more susceptible to the cytotoxic effects of cisplatin by pretreatment with the 1,25-D3 analogue Ro23–7553 (18). Given our current findings, it is tempting to speculate that the enhanced cytotoxicity of cisplatin in SCC observed after Ro23–7553 treatment is due to 1,25-D3-mediated down-modulation of p21 expression in these cells. Experiments to test this hypothesis are now in progress.

Exposure of SCC cells to 1,25-D3 for 24 h in vitro resulted in a ~2-fold decrease in p21 transcripts (Fig. 3A). This may account, in part, for the 3-fold reduction in p21 protein levels observed after 1,25-D3 administration (Fig. 2A). Although 1,25-D3 has previously been shown to activate p21 transcription in U937 cells (5), the VDR has been reported to repress the transcription of certain genes, including interleukin-2 (42) and granulocyte macrophage colony-stimulating factor (43). It is, therefore, theoretically possible that the transcript reduction we observe is due to a direct, inhibitory effect of 1,25-D3 on p21 transcription initiation. However, it is likely that p21 levels are regulated by additional posttranscriptional mechanisms in SCC because treatment with 1,25-D3, in combination with dexamethasone results in only a 2-fold decrease in p21 transcripts, but a nearly 10-fold decrease in p21 protein levels (Figs. 2A and 3A). Recent studies indicate that p21 is degraded by a proteosome-dependent pathway (44); 1,25-D3 treatment may activate this endogenous mechanism and, thereby, indirectly down-modulate p21.

Our data are consistent with a model in which 1,25-D3 exerts antiproliferative activity in SCC by modulating expression of the cdk inhibitors p21 and p27. Significantly, modulation of p21 by 1,25-D3 occurs both in tumor cells treated in tissue culture and in tumors isolated from treated mice. In vitro, 1,25-D3 administration results in G0-G1 arrest that is associated with increased expression of p27 and decreased expression of p21. In vivo, 1,25-D3 administration results in tumor regression (presumably via the induction of cell death) that is associated with decreased expression of p21 and no change in p27 expression. On the basis of these data, we propose that the mechanism by which 1,25-D3 exerts antiproliferative activity in SCC may differ in vivo and in vitro and that the decision between cell cycle arrest or cell death may depend critically on the balance between p21 and p27 expression.

ACKNOWLEDGMENTS

We thank Dr. Richard Steinman for helpful comments and suggestions provided during the course of this work.

REFERENCES


Table 2 1,25-D3 treatment modulates SCC expression of p21 in vivo

<table>
<thead>
<tr>
<th>Time</th>
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<td>1,25-D3</td>
<td>213</td>
<td>245</td>
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Values determined to be statistically different from control using the Student’s t test (P < 0.01) are in bold. Similar results were obtained in a second independent experiment.


1,25-Dihydroxycholecalciferol (1,25-D$_3$) Inhibits the Growth of Squamous Cell Carcinoma and Down-Modulates p21 Waf1/Cip1 in Vitro and in Vivo
