Published in final edited form as:
Mol Cancer Ther. 2008 September ; 7(9): 3047–3055. doi:10.1158/1535-7163.MCT-08-0243.

$1,25D_3$ potentiates cisplatin antitumor activity by p73 induction in a squamous cell carcinoma model

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Abstract

$1,25D_3$ exhibits anti-tumor activity in a variety of cancers including squamous cell carcinoma (SCC). Intrinsic resistance of SCC cells to cisplatin was observed and led to the investigation into whether $1,25D_3$ sensitizes SCC cells to cisplatin. Pretreatment with $1,25D_3$ followed by cisplatin enhanced growth inhibition in SCC cells compared with $1,25D_3$ alone, as assessed by cytotoxicity and *in vitro* clonogenic assays. In addition, $1,25D_3$ sensitized SCC cells to cisplatin-mediated apoptosis. Treatment of tumor-bearing C3H mice with $1,25D_3$ prior to cisplatin reduced clonogenic survival using *in vivo* excision clonogenic assay. These results were not observed in a $1,25D_3$-resistant SCC variant, indicating the critical role of $1,25D_3$ in sensitizing SCC cells to cisplatin. Further, a marked decrease in fractional tumor volume was observed when SCC tumor-bearing mice were treated with $1,25D_3$ prior to cisplatin as compared to either agent administered alone. Cisplatin has been shown to modulate p73 protein level in certain cancer cells. Our data showed that p73 level was not affected by cisplatin, but increased by $1,25D_3$ in SCC cells. Knocking down p73 by siRNA protected SCC cells against $1,25D_3$ and cisplatin-mediated clonogenic cell kill and apoptosis. Increasing p73 protein level by knocking down UFD2a, which mediates p73 degradation, promoted $1,25D_3$ and cisplatin-mediated clonogenic cell kill. These results suggest that $1,25D_3$ potentiates cisplatin anti-tumor activity *in vitro* and *in vivo* in a SCC model system, possibly through p73 induction and apoptosis. The combination treatment may provide a more effective therapeutic regimen in cancer treatment.

Keywords

$1,25D_3$; p73; cisplatin

Introduction

Vitamin D regulates diverse physiological functions including calcium homeostasis, bone metabolism, cell differentiation and immunity (1,2). 1α,25-dihydroxyvitamin D_3 ($1,25D_3$), the most active metabolite of vitamin D, inhibits the growth of a number of cancer types such as prostate, breast, colorectal, ovarian, and skin cancers (1,2). $1,25D_3$ is currently being evaluated, alone or in combination with other chemotherapeutic agents, in clinical trials for the treatment of several solid tumors (1,3).

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Cisplatin (*cis*-diammine-dichloro-platinum (II), cDDP) is a potent chemotherapeutic agent widely used for the treatment of a variety of cancers, including testicular, ovarian, cervical, lung cancer and head and neck squamous cell carcinoma (SCC) (4,5). However, its effectiveness as an anti-cancer agent is limited by drug resistance and side effects including nephrotoxicity, emetogeneiss and neurotoxicity (6). Tumor resistance to cisplatin may be caused by insufficient DNA binding, increased DNA repair ability, bypass of DNA adducts, or impaired apoptosis (7). Hence, it will be beneficial if tumor cells can be sensitized to cisplatin treatment with a combination therapy.

DNA damage caused by cisplatin may induce the activation of tumor suppressor p53 (6,8), which inhibits cell proliferation by promoting cell cycle arrest or apoptosis. The presence of wild type p53 correlates to the sensitivity to cisplatin (6). Since p53 is frequently mutated or functionally impaired in human cancers, the status of a p53 related protein, p73, is considered to be an important determinant of cellular sensitivity to chemotherapeutic drugs.

p73 has significant homology to p53. p73 gene encodes multiple isoforms due to the usage of alternative promoters and the alternative splicing (9). Transcription of p73 gene from promoter P1 results in the isoforms containing an N-terminal transactivation (TA) domain (TAp73), whereas the isoforms transcribed from promoter P2 are N-terminal truncated and lack the TA domain (ΔNp73) (9). TAp73 is a transcription factor and regulates genes involved in cell cycle arrest and apoptosis and other cellular functions. Some genes are common targets of p53, such as Bax, Puma, and Noxa, while others are not regulated by p53. In contrast, ΔNp73 may serve as dominant negative inhibitors of p53 family (10).

Unlike p53, p73 mutation is rare in human cancers (10). Loss of heterozygosity and methylation-mediated gene silencing are observed in many cancer types (10). In addition, p73 gene polymorphism is implicated in tumorigenesis (11). p73 protein expression is deregulated in many cancers (10,11). Loss of p73 has been reported to associate with tumor progression and poor prognosis in several cancers (12–15). p73 loss triggers the conversion of kerotinocytes to SCC (16). Although p73 knockout mice do not develop spontaneous tumors in the initial studies, mice heterozygous for p73 ($p73^{+/-}$) or p63 ($p63^{+/-}$) develop malignant tumors at high frequency (17). Moreover, higher tumor burden and metastasis are observed in $p53^{+/-}$; $p73^{+/-}$ and $p53^{+/-}$; $p63^{+/-}$ mice compared to $p53^{+/-}$ mice (17). These observations indicate p73 plays a role in tumor development.

We previously demonstrated that $1,25D_3$ exerts anti-proliferative effects in murine SCC cell line SCCVII/SF (18–20). These effects are mediated by the induction of cell cycle arrest and apoptosis (20,21). We also demonstrated that pretreatment with $1,25D_3$ enhances paclitaxel, cisplatin, or carboplatin-mediated antitumor activities (22–24). However, the mechanisms for $1,25D_3$-enhanced cisplatin antitumor effects remain unclear.

In the current study, we established a variant of SCC cell line, SCC-DR, which is resistant to $1,25D_3$ and thereby serves as a control to study the effects of $1,25D_3$. Further, we investigate the mechanisms of $1,25D_3$ and cisplatin-mediated growth inhibition, especially the role of p73 and apoptosis in SCC cells.

Materials and Methods

Materials

$1,25D_3$ was a generous gift from Hoffmann-LaRoche (Nutley, NJ). Cisplatin (Platinol-AQ) was obtained from Bristo-Myers Squibb Company (Princeton, NJ). Anti-VDR (sc-1008) and anti-phosphorylated ERK1/2 (sc-7383) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase 3 (9662), anti-caspase 8 (4927), anti-caspase 9 (9504), anti-caspase 10

(9752), anti-phosphorylated Akt (Ser^{473}; 9271), anti-Akt (9272), anti-ERK1/2 (9102) and anti-p53 (2524) were from Cell Signaling Technology (Beverly, MA). Anti-p73 (IMG-246, clone 5B429) was from Imgenex (San Diago, CA). Anti-PARP (556362) and anti-p63 (550025) was from BD Pharmingen. Anti-actin (CP-01) was from Calbiochem (San Diego, CA).

Cell culture and tumor model systems

Murine SCC (SCCVII-SF) is a moderately well-differentiated SCC derived from a spontaneously arising tumor of the C3H mouse (25). SCC cells were maintained in 6–10 weeks old female C3H/HeJ mice from Jackson Laboratory (Bar Harbor, ME). SCC cells were cultured in RPMI 1640 media supplemented with 12% FBS and 1% penicillin/streptomycin sulfate. The mice protocols used for *in vivo* excision clonogenic assays were approved by the Roswell Park Cancer Institutional Animal Care and Use Committee. The mice protocols used for tumor regrowth delay were approved by University of Pittsburgh animal care committee according to USPHS guidelines.

Generation of SCC-DR cells

SCC cells were continuously cultured in RPMI 1640/FBS media containing 10 nM of $1,25D_3$ for over 10 months until no cytotoxicity was observed on a light microscope. The resulting stable SCC-DR cell line was maintained in RPMI 1640/FBS containing 10 nM of $1,25D_3$. For experiments, SCC-DR cells were plated in RPMI 1640/FBS without $1,25D_3$ over night and subjected to further treatment.

Trypan blue exclusion assay

Cell viability was quantitatively assessed by Trypan blue exclusion assay using Vi-CELL™ Series Cell Viability Analyzers (Beckman-Coulter, Fullerton, CA).

Cytotoxicity assay

Cytotoxicity was quantified by the released lactate dehydrogenase (LDH) from the cytosol of damaged cells using Cytotoxicity Detection Kit^{PLUS} (LDH) kit following the manufacturer's protocol (Roche Applied Science, Indianapolis, IN).

In vitro Clonogenic assay

SCC or SCC-DR cells were pretreated with ethanol (ETOH) or 10 nM $1,25D_3$ for 24 h, and then treated with 0.5 μg/ml cisplatin for 2 h or left untreated. Cisplatin was then washed away and $1,25D_3$ was replaced in the groups treated with $1,25D_3$. The *in vitro* clonogenic assays were performed as described (23,26).

In Vivo Clonogenic Assay

The *in vivo* effects of $1,25D_3$ and cisplatin on clonogenic SCC cells were determined by *in vivo* excision clonogenic assay as described (22,26–28). Briefly, C3H mice bearing 9-day SCC or SCC-DR tumors were treated in 4 groups (3 to 5 per group): saline, $1,25D_3$, cisplatin, or $1,25D_3$ and cisplatin combination. Mice were treated for 3 d with daily i.p. injection of saline or 0.625 μg/mouse of $1,25D_3$. On day 3, mice also received i.p. injection of 3 mg/kg of cisplatin. Twenty-four h after the last injection, mice were sacrificed, and their tumors were excised. Clonogenic assays were performed as described (26).

Tumor regrowth delay

SCC cells (4.5×10^5) were inoculated s.c. into the flank of the C3H mice. Studies were initiated when the tumors were palpable. Mice were treated in 4 groups (10 per group): saline, $1,25D_3$, cisplatin, or $1,25D_3$ and cisplatin combination. Mice were treated for 3 d with single,

daily i.p. injections of saline or 0.25 μg/mouse of i.p. injection of 6 mg/kg of cisplatin. $1,25D_3$. On day 3, mice also received a singleTumor measurements were done as described (22).

Immunoblot analysis

Cell lysates were prepared and immunoblot analysis was performed as described previously (19,21).

Apoptosis assay - DNA fragmentation ELISA

SCC cells were harvested, lysed, and DNA fragmentation was quantitatively evaluated by Cell Death Detection ELISAPLUS according to the manufacturer's instructions as described (19, 21).

Real-time quantitative RT-PCR

Total RNA from SCC cells was isolated using the RNeasy Mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg total RNA using oligo(dT) primers (iScript cDNA synthesis kit, Bio-Rad, Hercules, CA). Gene quantification was performed on an Applied Biosystems 7300 real-time system (Applied Biosystems, Foster City, CA) with standard thermal cycler conditions. TaqMan primers and probes for p73 and GAPDH were purchased from Applied Biosystems. Relative gene expression was determined by the $\Delta\Delta$-C_T method.

siRNA transfection

Synthetic small interfering RNA (siRNA) siGENOME SMARTpool siRNAs (4 individual siRNA pooled together) specific for p73, UFD2a, siCONTROL non-specific siRNA (siRNA-NS), and DharmaFECT1 transfection reagent were from Dharmacon (Lafayette, CO). SCC cells were transfected with 50 nM siRNA-NS or siRNA against p73 or UFD2a for 24 h using DharmaFECT1 transfection reagent following the manufacturer's protocol.

Statistics

Statistical significances between groups were determined by two-tailed student's *t*-test.

Results

Generation of $1,25D_3$ resistant SCC cells

We previously demonstrated that $1,25D_3$ has antitumor effects in SCC cells (18–20). Cisplatin is widely used to treat patients with head and neck SCC with moderate success (29). Therefore, SCC cell line serves as an ideal model to study the effects of combination treatment with $1,25D_3$ and cisplatin.

We previously reported that $1,25D_3$ induces cell cycle arrest and apoptosis in SCC cells (19, 20,30). However, only a small percentage of the cells responded to $1,25D_3$. To have a better understanding on the effects of $1,25D_3$ in SCC cells, we established a $1,25D_3$-resistant variant of SCC, SCC-DR cell line, by continuously culturing SCC cells in media containing 10 nM of $1,25D_3$, the dose that has anti-proliferative effects in SCC cells and is clinically achievable in man (19,20,30). To examine whether SCC-DR cells are resistant to the growth inhibitory effects of $1,25D_3$, SCC-DR cells or control parental SCC cells were treated with 10 or 500 nM of $1,25D_3$ and subjected to *in vitro* clonogenic assay. The colony-forming capacity of SCC cells was greatly inhibited by $1,25D_3$ (Fig. 1A). In contrast, the colony-forming capacity of $1,25D_3$-treated SCC-DR cells was mostly intact (Fig. 1B).

To further characterize the cellular functions of SCC-DR cells, $1,25D_3$-mediated transcriptional activity and apoptosis were examined. No induction of vitamin D receptor (VDR) was observed in SCC-DR cells until 1000 nM of $1,25D_3$ was used, while VDR was induced in SCC cells upon 10 nM $1,25D_3$ treatment (Fig. 1B), suggesting that SCC-DR cells have compromised transcriptional activity of VDR. Additionally, pro-caspase 3 was readily cleaved in SCC cells with 10 nM $1,25D_3$ treatment while it remained intact in SCC-DR cells (Fig. 1B); suggesting that SCC-DR cells are resistant to $1,25D_3$-induced apoptosis. We previously demonstrated that $1,25D_3$ induces nongenomic activation (occurs within 5 min) of Akt and ERK1/2 in SCC cells (21). $1,25D_3$ did not induce rapid activation of Akt in SCC-DR cells (Fig. 1C). Interestingly, $1,25D_3$ activated ERK1/2 in SCC-DR cells at ~ 30 min (Fig. 1C), indicating the nongenomic signaling of $1,25D_3$ is partially affected. Together, these results show that SCC-DR cells are resistant to $1,25D_3$ treatment at several aspects, therefore, it may serve as a control model to study the effects of $1,25D_3$ in sensitive cell lines.

SCC and SCC-DR cells are resistant to cisplatin

To examine whether cisplatin has cytotoxic effects in SCC or SCC-DR cells, the cells were treated with various doses (0 to 1 μg/ml) of cisplatin for 48 h and cell viability was assessed by Trypan blue exclusion assay. Surprisingly, cisplatin had no cytotoxic effects in SCC and SCC-DR cells even at 1 μg/ml, suggesting they are resistant to cisplatin over the range of concentration tested (Fig. 2A).

$1,25D_3$ sensitizes SCC cells to cisplatin treatment *in vitro*

We previously demonstrated that $1,25D_3$ and cisplatin have synergistic grow inhibition in SCC cells indicated by MTT assays (24). To further determine whether $1,25D_3$ can sensitize SCC cells to cisplatin, two other methods were employed, cytotoxicity assay by measuring the released lactate dehydrogenase from damaged cells and the *in vitro* clonogenic assay. SCC or SCC-DR cells were pretreated with 10 nM $1,25D_3$ or vehicle control ETOH for 24 h followed by 0.5 μg/ml cisplatin or control media for 2 h. Cytotoxicity was assessed after an additional 48 h of incubation. $1,25D_3$ induced significant ($P < 0.01$) cytotoxicity in SCC cells (Fig. 2B). Cisplatin did not induce cytotoxicity, however, pretreatment of $1,25D_3$ for 24 h followed by cisplatin resulted in enhanced cytotoxicity compared with $1,25D_3$ alone, suggesting that $1,25D_3$ sensitized SCC cells to cisplatin-induced cell killing (Fig. 2B). In contrast, cytotoxicity was not observed upon any treatment in SCC-DR cells (Fig. 2B). The more sensitive clonogenic assay revealed that $1,25D_3$ or cisplatin alone markedly inhibited the clonogenic capacity of SCC cells. The combination treatment had more profound effect than $1,25D_3$ or cisplatin alone (Fig. 2C). $1,25D_3$ alone did not alter the clonogenic capacity of SCC-DR cells (Fig. 2C), while cisplatin or the combination modestly suppressed the clonogenic ability of SCC-DR cells (Fig. 2C). These results suggest that $1,25D_3$ potentiates cisplatin anti-proliferative effects in SCC cells.

$1,25D_3$ promotes cisplatin anti-tumor activity *in vivo*

To evaluate whether $1,25D_3$ also enhances the anti-proliferative effects of cisplatin *in vivo*, the *in vivo* excision clonogenic assay was used. We previously showed that this assay is an indication of *in vivo* anti-tumor activity (23,27,28,31,32). SCC or SCC-DR tumor-bearing mice were treated with saline, 0.625 μg $1,25D_3$ daily for 3 d, 3 mg/kg cisplatin on day 3, or the combination of 0.625 μg $1,25D_3$ daily for 3 d and 3 mg/kg cisplatin on day 3. The combination of $1,25D_3$ and cisplatin resulted in a significantly greater decrease in surviving fraction as compared with $1,25D_3$ ($P < 0.01$) or cisplatin ($P < 0.0001$) alone compared with $1,25D_3$ (Fig. 3A). In contrast, $1,25D_3$ or cisplatin alone had no significant activity in SCC-DR cells (Fig. 3A), and the combination treatment resulted in a slight decrease in surviving fraction (Fig. 3A), suggesting critical role of $1,25D_3$ in clonogenic cell kill.

To determine the effects of $1,25D_3$ and cisplatin on tumor growth *in vivo*, SCC tumor-bearing daily for 3 d, 6 mg/kg cisplatin on mice were treated with saline, 0.25 μg $1,25D_3$day 3, or the combination of 0.25 μg $1,25D_3$ daily for 3 d with 6 mg/kg cisplatin on day dosing regimen was reported previously to maximize anti-tumor efficacy 3. This $1,25D_3$ while minimizing toxicity or hypercalcemia (23). $1,25D_3$ or cisplatin alone exhibited tumor-inhibitory effects in SCC as compared with the saline control (Fig. 3B). The combination of $1,25D_3$ and cisplatin resulted in enhanced tumor regression compared to single agent (Fig. 3B). Mice in saline, $1,25D_3$, and cisplatin treatment groups had to be enhances sacrificed early as a result of tumor burden. These results indicate that $1,25D_3$*in vivo* antitumor activity of cisplatin in the SCC model.

$1,25D_3$ promotes cisplatin to induce apoptosis

$1,25D_3$ induces apoptosis in SCC cells (19,21,30). $1,25D_3$ and cisplatin treatment led to increased caspase 3 cleavage compared to single agent treatment (24). To further characterize $1,25D_3$ and cisplatin-induced apoptosis, DNA fragmentation was evaluated by Cell Death Detection ELISA. $1,25D_3$ enhanced DNA fragmentation in SCC cells compared with controls, while cisplatin did not induce apoptosis (Fig. 4A). The combination treatment resulted in a significantly ($P < 0.01$) higher level of apoptosis compared to $1,25D_3$ alone (Fig. 4A). Immunoblot analysis showed that $1,25D_3$ induced the cleavage of pro-caspases 8, 10, 3 and Poly (ADP-ribose) polymerase (PARP) in SCC cells, while cisplatin did not (Fig. 4B). The combination treatment resulted in enhanced cleavage of pro-caspases 8, 10 and PARP (Fig. 4B). None of these were observed in SCC-DR cells (Fig. 4B). Pro-caspase 9 was not cleaved by any treatment (Fig. 4B). These results suggest that $1,25D_3$ promotes cisplatin to induce apoptosis through a caspase 8/10 – caspase 3 pathway in SCC cells.

$1,25D_3$-augmented p73 level contributes to cisplatin-induced growth inhibition

p73 is one of the p53 family members and may regulate apoptosis (10). Cisplatin has been reported to promote 73 protein accumulation in HCT116 cells (33). Therefore, we next examined whether $1,25D_3$ and cisplatin alter the protein levels of p73 and other p53 family members in SCC or SCC-DR cells. $1,25D_3$ alone or in combination with cisplatin enhanced full length TAp73 (p73) levels in SCC cells as assessed by immunoblot analysis using a monoclonal antibody recognizing TAp73 but not reacting to Np73 nor p53 (Fig. 5A). In contrast, $1,25D_3$ resulted in reduced p53 protein level and the combination treatment also reduced p53 level (Fig. 5A). $1,25D_3$ alone or in combination with cisplatin also reduced p63 levels (Fig. 5A). Cisplatin did not affect the levels of p53, p63 or p73 in SCC cells (Fig. 5A). p53, p63, and p73 levels were not affected by any of the treatment in SCC-DR cells (Fig. 5A). To determine whether p73 accumulation contributes to $1,25D_3$ and cisplatin-induced growth inhibition, p73 was knocked down by siRNA. Since the endogenous p73 level was low, the efficiency of the p73 gene silencing was assessed by quantitative Real-time PCR (Fig. 5B). Following siRNA-transfection, SCC cells were further treated with ETOH or 10 nM $1,25D_3$ for 24 h followed by 0.5 μg/ml of cisplatin for 2 h and additional 6-day incubation for the *in vitro* clonogenic assay. siRNA-p73 significantly ($P<0.001$) promoted the surviving fraction following the treatment of $1,25D_3$, cisplatin, or the combination (Fig. 5B). To determine whether augmenting p73 protein level further promotes the anti-proliferative effects of $1,25D_3$ and cisplatin, we targeted a U-box-type E3/E4 ubiquitin ligase UFD2a which promotes the degradation of p73 (34). Knocking down endogenous UFD2a by siRNA resulted in p73 accumulation in SCC cells (Fig. 5C). With siRNA-UFD2a transfection, $1,25D_3$ or combination treatment resulted in significantly reduced surviving fraction when compared to the non-specific siRNA transfection (Fig. 5C). These data indicate that p73 contributes to the anti-proliferative effects of $1,25D_3$ and cisplatin.

p73 contributes to $1,25D_3$ and cisplatin-induced apoptosis

To further elucidate the mechanisms for $1,25D_3$ and cisplatin-induced growth inhibition, whether p73 plays a role in apoptosis was examined. Following siRNA transfection, SCC cells were treated with $1,25D_3$ and/or cisplatin and DNA fragmentation was evaluated after an additional 48 h of incubation. siRNA-p73 transfection resulted in reduced DNA fragmentation induced by $1,25D_3$ alone or $1,25D_3$ and cisplatin compared to controls (Fig. 6A). These results indicate that p73 contributes to $1,25D_3$ and cisplatin-mediated apoptosis.

Altogether, our data showed that $1,25D_3$ sensitizes SCC cells to cisplatin-induced growth inhibition by the induction of p73 which promotes apoptosis through a caspase 8/10-caspase 3 dependent pathway (Fig. 6B).

Discussion

$1,25D_3$ exerts anti-tumor effects *in vitro* and *in vivo* through inhibition of proliferation, induction of differentiation and apoptosis, and suppression of invasiveness of cancer cells (1). $1,25D_3$ has also been shown to synergistically or additively enhance the antitumor activities of a number of chemotherapeutic agents including carboplatin, cisplatin, docetaxel and paclitaxel in prostate cancer, breast cancer, and SCC models (22,23,35,36). The mechanisms for the enhanced anti-tumor effects are not well understood. $1,25D_3$ promoted caspase 3 cleavage when used in combination with cisplatin in SCC cells (24). $1,25D_3$ potentiates antitumor activity of paclitaxel by reducing p21 in PC3 cells (22). In addition, $1,25D_3$ promotes docetaxel-induced growth inhibition by reducing multidrug resistance-associated protein 1 (36). $1,25D_3$ has been shown to enhance the cytotoxicity of carboplatin when used in clinical trials in patients with prostate cancer and advanced cancer (3).

To better understand the role of $1,25D_3$ in SCC cells, $1,25D_3$-resistant SCC variant was generated. SCC-DR cells showed resistance to $1,25D_3$-mediated growth inhibition and apoptosis, and compromised VDR transcription activity and nongenomic signaling.

Cisplatin is a widely used chemotherapeutic agent. Unfortunately, drug resistance and toxic side effects limit its usage. Therefore, if tumor cells can be sensitized to cisplatin treatment, lower and thus more tolerated dose can be used in the treatment. Potential mechanisms for acquired resistance to cisplatin include drug inactivation by glutathione and metallothionein, enhanced DNA repair, decreased cisplatin accumulation, increased cisplatin adducts tolerance and impaired apoptotic pathway (6). We previously showed that cellular concentration of cisplatin and cisplatin-DNA adducts did not change in response to $1,25D_3$ and cisplatin combination treatment compared to cisplatin alone (24).

Although we previously demonstrated that $1,25D_3$ enhanced cisplatin antiproliferative effects and caspase 3 cleavage in SCC cells (24), the mechanisms for these effects are largely unknown. Our current study identified p73 as a target of $1,25D_3$, the level of which is increased upon $1,25D_3$ treatment. We further demonstrate that p73 contributes to $1,25D_3$ and cisplatin-mediated growth inhibition.

We show that SCC and SCC-DR cells are resistant to cisplatin, and $1,25D_3$ sensitizes SCC cells to cisplatin-induced growth inhibition. Pretreatment with $1,25D_3$ followed by cisplatin resulted in enhanced clonogenic cell kill in SCC, but not SCC-DR, cells *in vitro* and *in vivo*. $1,25D_3$ in combination with cisplatin suppressed SCC tumor growth compared with either agent administered alone. This is in line with our previous data showing that a vitamin D analog, Ro23-7553, increased tumor regrowth delay in a combination therapy with cisplatin when compared to either agent administered alone (23). Cisplatin alone does not induce apoptosis in SCC cells, while pretreatment with $1,25D_3$ followed by cisplatin greatly enhances apoptosis

compared to $1,25D_3$ alone. Others have shown that damaged apoptotic pathway is one of the mechanisms for cisplatin resistance (6,37). Impaired apoptosis may involve dysregulation and mutations of apoptosis-mediating molecules which result in the inability of cells to detect DNA damage or to induce apoptosis (38–40). Therefore, SCC cells may be resistant to cisplatin treatment because cisplatin alone fails to induce apoptosis. When SCC cells are pretreated with $1,25D_3$, the apoptotic pathway is restored and cisplatin is able to further promote apoptosis, which is indicated by enhanced cleavage of pro-caspase 10 and PARP and increased DNA fragmentation.

Cisplatin may induce apoptosis through the regulation of p53 family member p73, which is regulated by DNA damage, oncogenes, and viral proteins (10). Cisplatin enhances p73 level in HCT116 cells by stabilizing p73 protein (33). In addition, cisplatin-mediated p73 accumulation contributes to cisplatin-induced apoptosis in Hep3B cells (41). When overexpressed, p73 promotes cisplatin-induced apoptosis in HeLa cells (42). Surprisingly, cisplatin did not induce p73 in SCC cells in this study, which may be one of the reasons why cisplatin alone failed to induce apoptosis in SCC cells. In contrast, $1,25D_3$ alone or in combination with cisplatin enhanced p73 protein level in SCC cells, most likely through increasing the stability of p73, since $1,25D_3$ did not alter the mRNA level of p73 as shown by quantitative Real-time RT-PCR. $1,25D_3$ did not sensitize SCC-DR cells to cisplatin treatment. Further, p73, p53, and p63 levels were not affected by $1,25D_3$ in SCC-DR cells. These results indicate that $1,25D_3$ signaling plays a critical role in potentiating the growth inhibitory effects of cisplatin. When p73 is knocked down by siRNA approach, $1,25D_3$ and cisplatin-induced growth inhibition and apoptosis were suppressed. The endogenous protein level of p73 is very low in SCC cell cultures. The stability of p73 is regulated by the proteasome through ubiquitin-dependent and ubiquitin-independent pathways (43). UFD2a, a U-box-type ubiquintin protein ligase, has recently been reported to interact with and promote the degradation of p73 in a ubiquitin-independent manner (34). It does not affect the half life of p53 (34). We took advantage of this phenomenon and augmented p73 protein level by siRNA-UFD2a. Increased p73 level promoted $1,25D_3$ and cisplatin-induced growth inhibition in SCC cells. These results suggest that p73 contributes to the anti-proliferative and pro-apoptotic effects of $1,25D_3$ and cisplatin. In line with this concept, two recent studies show that p73 induction sensitizes tumor cells to therapies through enhanced apoptosis. CD154 sensitizes leukemia cells to fludarabine treatment via the activation of p73 and the consequent overcoming of the resistance to apoptosis (44). Endogenous expression of p73 was observed only in the radiosensitive cervical cancer cells, and p73 transfection in the radioresistant cells resulted in enhanced cellular sensitivity to radiation by increase of apoptosis (45).

The mechanisms for p73-induced apoptosis remain to be fully understood. p73 may induce apoptosis through the mitochondrial pathway by inducing Puma which causes Bax mitochondrial translocation and cytochrome c release in Saos-2 cells (46). This apoptosis can be inhibited by the ΔNp73 isoform (46). The induction of cyclin-dependent kinase inhibitor $p57^{kip2}$ is required for p73-mediated apoptosis in H1299 cells (47). Another study shows that p73 transcriptionally promotes the expression of death receptor Fas and sensitizes cells to apoptosis via a caspase-dependent pathway (48). Further studies are required to elucidate the mechanisms for p73-mediated apoptosis in SCC cells.

In summary, the current study demonstrates for the first time that $1,25D_3$ increased p73 protein level in SCC cells which sensitized SCC cells to cisplatin-mediated growth inhibition and apoptosis. We propose that the combination of $1,25D_3$ and cisplatin as a strategy to overcome cisplatin resistance and dose limitation.

Acknowledgements

Financial support: This study was supported by NIH/NCI grants CA67267 and CA85142 to Dr. Candace S. Johnson, and CA95045 to Dr. Donald L. Trump.

Abbreviations list

$1,25D_3$
: 1α,25-dihydroxyvitamin D_3

cDDP
: cisplatin, *cis*-diammine-dichloro-platinum (II)

ETOH
: ethanol

LDH
: lactate dehydrogenase

PARP
: poly (ADP-ribose) polymerase

SCC
: squamous cell carcinoma

TA
: transactivation

VDR
: vitamin D receptor

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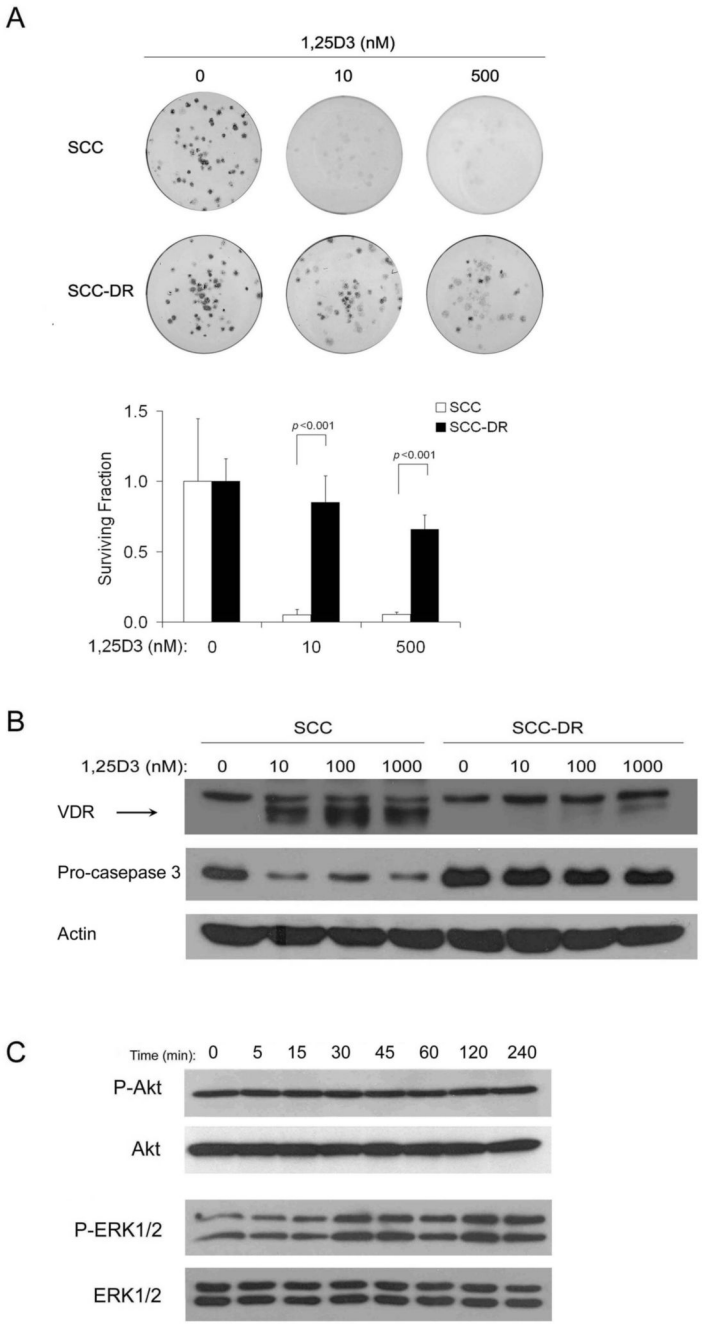

Figure 1.

SCC-DR cells are resistant to $1,25D_3$ treatment. **A,** SCC or SCC-DR cells were treated with 0, 10, or 500 nM of $1,25D_3$ and subjected to *in vitro* clonogenic assay. After staining, colonies were viewed and counted on a light microscope and photographed. Surviving fraction was calculated by dividing the cloning capacity of treated cells to that of ETOH control. Results of the surviving fraction are the mean ± SD of triplicate experiments and are representative of two independent experiments. **B,** SCC and SCC-DR cells were treated with 0 to 1000 nM of $1,25D_3$ for 48 h, and the levels of VDR and caspase 3 were assessed by immunoblot analysis. Actin was the loading control. Results are representative of two independent experiments. (**c**) SCC-DR cells were treated with 10 nM of $1,25D_3$ for 5 to 240 min, and the levels of

phosphorylated Akt and ERK1/2 were evaluated by immunoblot analysis. Total Akt or ERK1/2 level was assessed as the loading control.

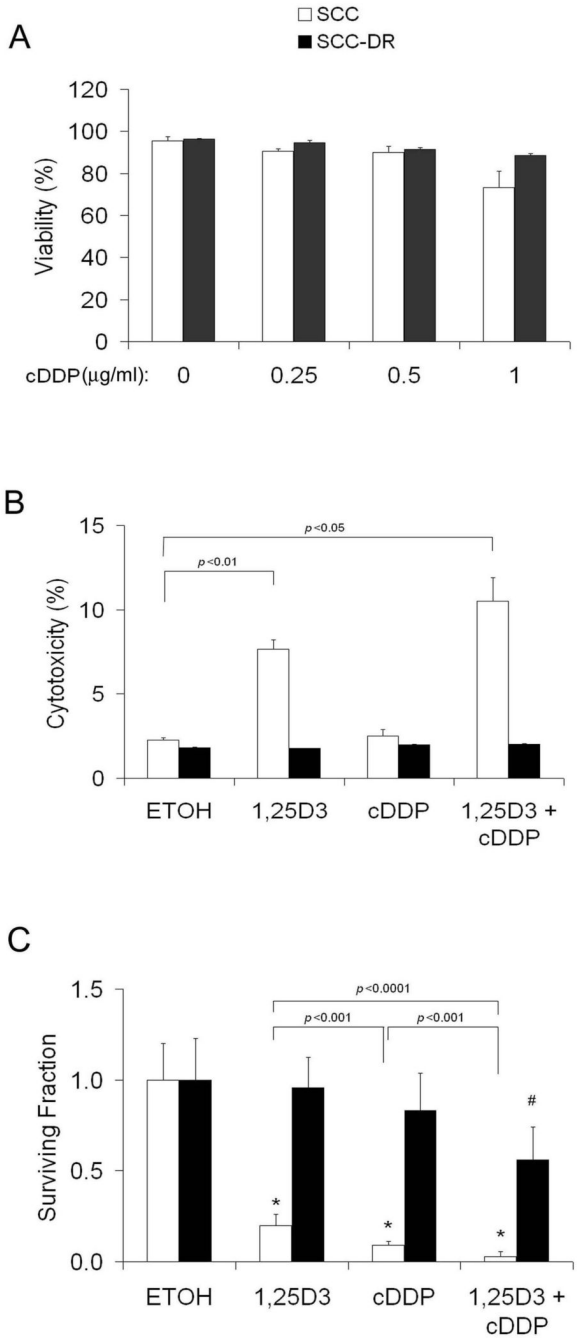

Figure 2.

$1,25D_3$ sensitizes SCC cells to cisplatin treatment. **A,** SCC or SCC-DR cells were treated with 0 to 1 μg/ml of cisplatin for 48 h, and cell viability was assessed by Trypan blue exclusion assay. Results are the mean ± SD of triplicate experiments and are representative of two independent experiments. **B,** SCC or SCC-DR cells were pretreated with vehicle control ETOH or 10 nM $1,25D_3$ for 24 h followed by 0.5 μg/ml of cisplatin for 2 h. Cells were harvested after an additional 48 h of incubation. Cytotoxicity was examined by LDH Cytotoxicity Detection Kit. **C,** Various dilutions of SCC or SCC-DR cells were plated in six-well tissue culture plates over night. They were pretreated with ETOH or 10 nM $1,25D_3$ for 24 h, and then incubated without further treatment or 0.5 μg/ml cDDP for 2 h and subjected to *in vitro* clonogenic assay.

Results are representative of two to three independent experiments. *cDDP*, cisplatin. *, $P < 0.00001$, vs. ETOH; #, $P < 0.01$, vs. cDDP.

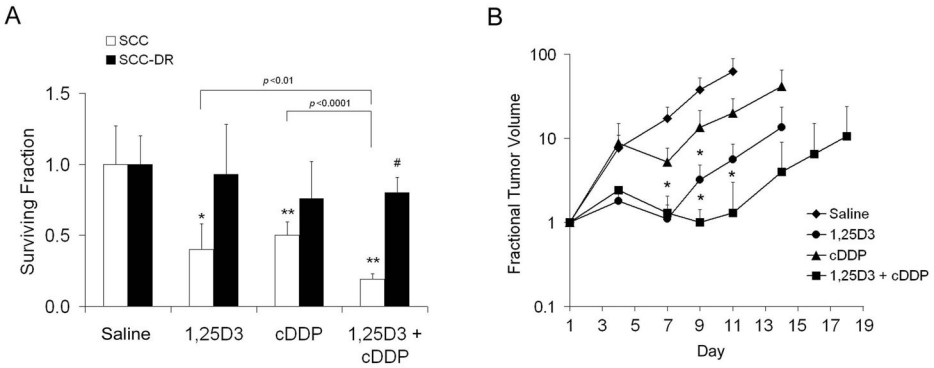

Figure 3.

$1,25D_3$ promotes cisplatin anti-tumor activity in SCC *in vivo*. **A,** SCC tumor- bearing mice (3 to 5 per group) were treated with saline or 0.625 μg of $1,25D_3$ daily for 3 d. On day 3, mice also received 3 mg/kg of cisplatin. Both agents were administered i.p. The *in vivo* excision clonogenic assay was performed 24 h after the last treatment. Each point represents the mean surviving fraction for total clonogenic cells per gram of tumor. *, $P < 0.001$, **, $P < 0.0001$, vs. saline; #, $P < 0.01$, vs. saline. **B,** C3H mice (10 per group) bearing palpable subcutaneous SCC tumors were treated with either saline, 0.25 μg of $1,25D_3$ daily for 3 d, 6 mg/kg of cisplatin on day 3, or the combination of 0.25 μg of $1,25D_3$ daily for 3 d and 6 mg/kg of cisplatin on day 3. Both agents were administered i.p. Tumor measurements were obtained on the days indicated, and fractional tumor volumes were calculated as described in Methods. Data points represent the mean ± SD fractional tumor volume for 10 mice/group. *, $P < 0.05$. *cDDP*, cisplatin.

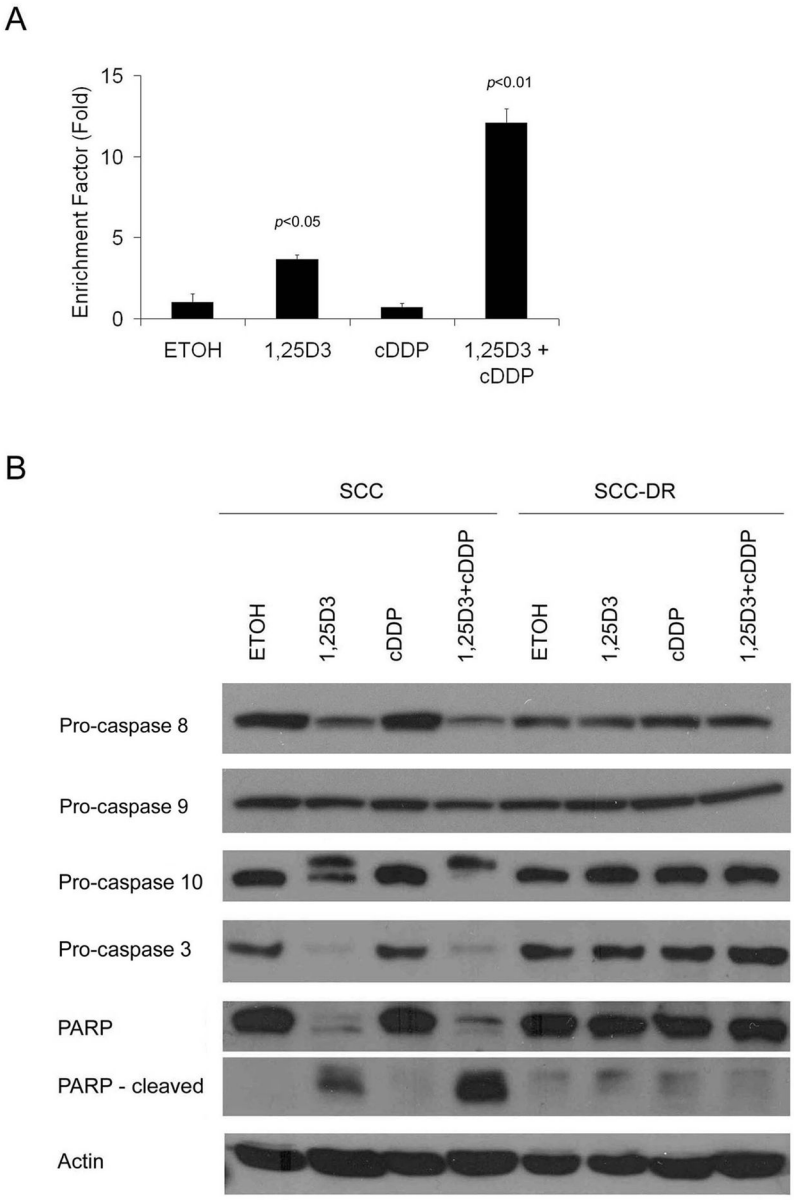

Figure 4.

$1,25D_3$ promotes cisplatin to induce apoptosis. **A,** SCC cells were pretreated with ETOH or 10 nM $1,25D_3$ for 24 h followed by 0.5 μg/ml of cisplatin for 2 h and an additional 48 h of incubation. Cells were harvested, lysed, and DNA fragmentation was evaluated by Cell Death Detection ELISA^{PLUS} according to the manufacturer's protocol. The enrichment factor was used as a parameter of apoptosis and shown on the *y* axis as mean ± SD of triplicate experiments. **B,** SCC or SCC-DR cells were pretreated with vehicle control ETOH or 10 nM $1,25D_3$ for 24 h followed by 0.5 μg/ml of cisplatin for 2 h. After an additional 48 h of incubation, cells were harvested and pro-caspases 8, 9, 10, 3 and PARP levels were evaluated by immunoblot analysis. Actin was the loading control. Results are representative of three independent experiments. *cDDP*, cisplatin.

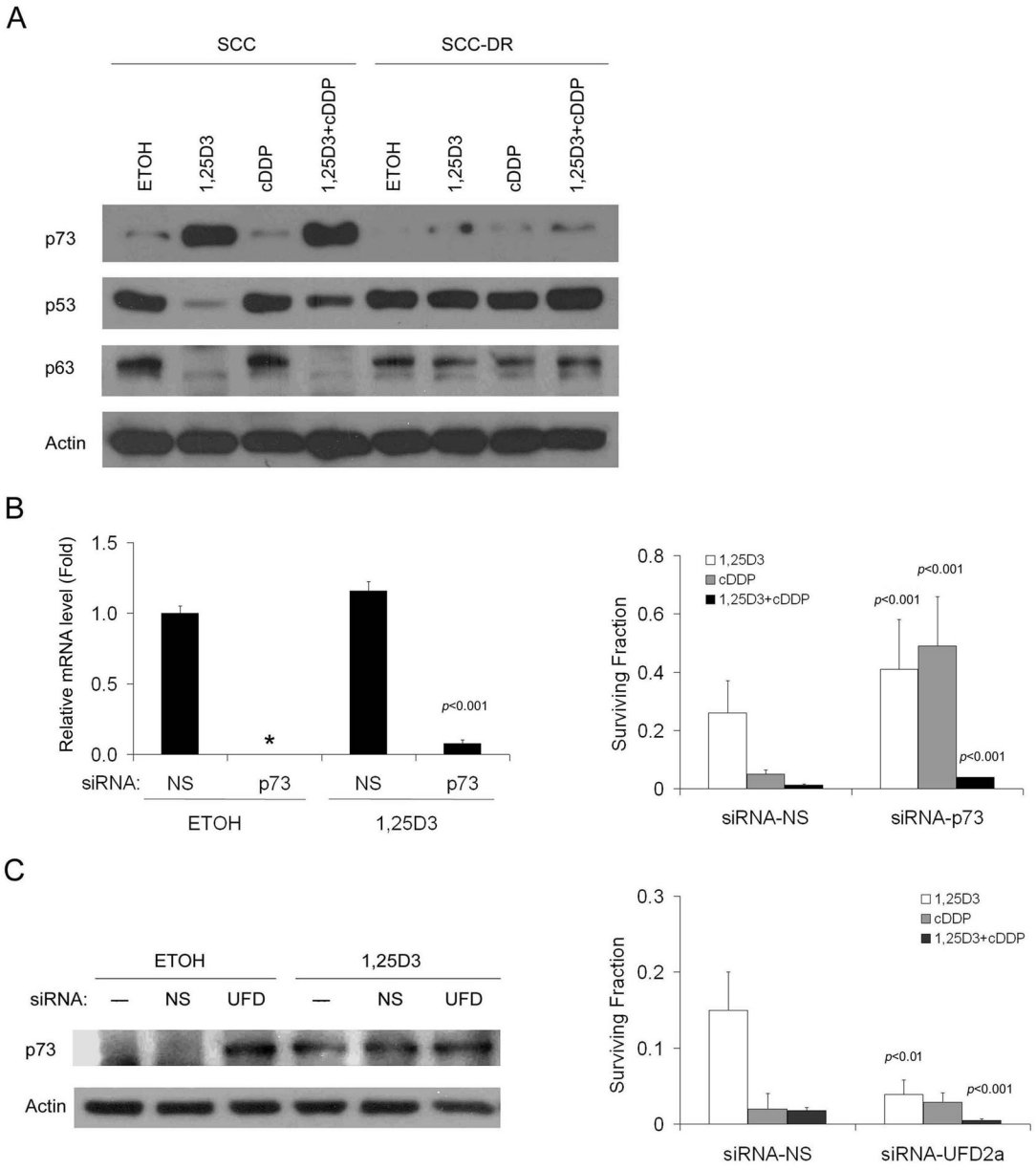

Figure 5.

$1,25D_3$-incrased p73 protein level contributes to cisplatin-induced growth inhibition. **A,** SCC or SCC-DR cells were pretreated with ETOH or 10 nM $1,25D_3$ for 24 h followed by 0.5 μg/ml of cisplatin for 2 h and an additional 48 h of incubation. Cells were harvested and p53, p63 and p73 levels were evaluated by immunoblot analysis. Actin was the loading control. **B,** SCC cells were transfected with siRNA-NS, siRNA- p73, or left untransfected for 24 h, followed by the treatment with $1,25D_3$ for 48 h. p73 mRNA level was evaluated by Real-time quantitative PCR. Results are the mean ± SD of the relative expression level to GAPDH. NS, non-specific; *, undetectable. Following siRNA-transfection, SCC cells were pretreated with ETOH or 10 nM $1,25D_3$ for 24 h followed by 0.5 μg/ml of cisplatin for 2 h. Anti-proliferative effect was assessed by *in vitro* clonogenic assay. Results are representative of two independent experiments. **C,** SCC cells were transfected with siRNA-NS, siRNA-UFD2a, or left untransfected for 24 h, followed by the treatment with $1,25D_3$ for 48 h. p73 protein level was

evaluated by immunoblot analysis. Actin was the loading control. UFD, UFD2a. Following siRNA- transfection, SCC cells were pretreated with ETOH or 10 nM $1,25D_3$ for 24 h followed by 0.5 μg/ml of cisplatin for 2 h. Anti-proliferative effect was assessed by *in vitro* clonogenic assay. Results are representative of two independent experiments. *cDDP*, cisplatin.

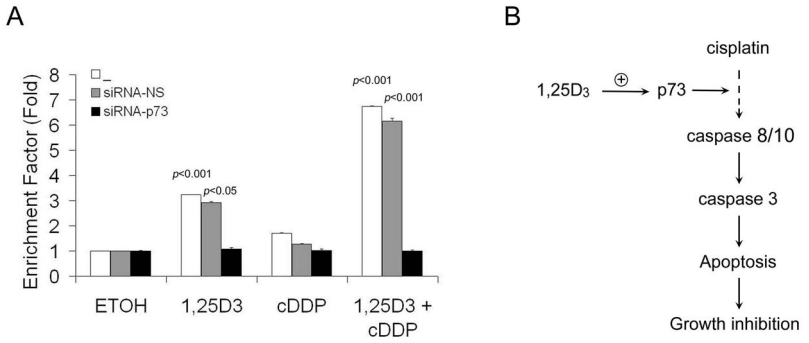

Figure 6.

A, p73 contributes to $1,25D_3$ and cisplatin-induced apoptosis. SCC cells were transfected with siRNA-NS, siRNA-p73, or left untransfected for 24 h. Cells were then pretreated with ETOH or 10 nM $1,25D_3$ for 24 h followed by 0.5 μg/ml of cisplatin for 2 h and an additional 48 h of incubation. Cells were harvested and DNA fragmentation was evaluated by Cell Death Detection ELISA^{PLUS} kit. The enrichment factor was used as a parameter of apoptosis and shown on the *y* axis as mean ± SD of triplicate experiments and are representative of three independent experiments. *cDDP*, cisplatin. **B,** A schematic presentation of the $1,25D_3$ potentiation of cisplatin anti-tumor activity. SCC cells are resistant to cisplatin treatment. $1,25D_3$ induces p73 accumulation and sensitizes SCC cells to cisplatin-mediated growth inhibition through caspase 8/10-caspase 3-dependent apoptotic pathway.