Cisplatin Potentiates 1,25-Dihydroxyvitamin D_3-induced Apoptosis in Association with Increased Mitogen-activated Protein Kinase Kinase Kinase 1 (MEKK-1) Expression[1]

Pamela A. Hershberger,[2, 3] Terence F. McGuire,[2] Wei-Dong Yu, Eleanor G. Zuhowski, Jan H. M. Schellens, Merrill J. Egorin, Donald L. Trump, and Candace S. Johnson

Departments of Pharmacology [P. A. H., T. F. M., W-D. Y., E. G. Z., M. J. E., C. S. J.], Medicine [M. J. E., D. L. T., C. S. J.], Urology [D. L. T.], and The University of Pittsburgh Cancer Institute [M. J. E., D. L. T., C. S. J.], University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, and Department of Experimental Therapy, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands [J. H. M. S.]

Abstract

1,25-Dihydroxyvitamin D_3 (1,25D_3) exhibits potent antitumor activity in the murine squamous cell carcinoma (SCC) SCCVII/SF, and the combination of 1,25D_3 with cisplatin (1,25D_3/cisplatin) demonstrates even greater activity. Because these agents possess different mechanisms of cytotoxicity, studies were initiated to define the mechanism by which the combination displays enhanced activity. Median dose-effect analysis demonstrates that 1,25D_3 and cisplatin act synergistically to inhibit SCC growth. When SCC cells were treated with 1,25D_3 (10 nM) and/or cisplatin (0.5 μg/ml), greater caspase-3 activation was observed for the combination than for either agent alone. This suggests that the enhanced cytotoxicity is, at least in part, due to greater induction of apoptosis. No alterations in cellular platinum concentration or platinum-DNA adducts were observed for 1,25D_3/cisplatin cotreatment compared with cisplatin treatment alone. Effects of the combination on cisplatin and 1,25D_3 signaling pathways in adherent (nonapoptotic) and floating (apoptotic) cells were explored. Cisplatin induced p53 and its downstream targets, $p21^{Cip1}$ (p21) and Bax, in both cell populations. In contrast, 1,25D_3 reduced p53, p21, and Bax to nearly undetectable levels in adherent cells. In the floating cells, 1,25D_3 reduced levels of p53 and p21, but Bax expression was maintained at control levels. Expression of these proteins in cells treated with 1,25D_3/cisplatin was similar to treatment with 1,25D_3 alone. The two agents also had divergent effects on survival and stress signaling pathways. Phospho-extracellular signal-regulated kinase 1/2 and phospho-Jun levels increased after treatment with cisplatin but decreased after treatment with 1,25D_3 and 1,25D_3/cisplatin. Moreover, cisplatin decreased levels of mitogen-activated protein kinase kinase kinase (MEKK-1), whereas 1,25D_3 up-regulated MEKK-1, and 1,25D_3/cisplatin further up-regulated MEKK-1. We propose that the increased cytotoxicity for 1,25D_3/cisplatin results from cisplatin enhancement of 1,25D_3-induced apoptotic signaling through MEKK-1.

Introduction

In addition to its classical effects on bone and mineral metabolism, calcitriol, or 1,25D_3,[4] has antiproliferative activity against a variety of solid tumors including those of the breast, prostate, and colon (1–5). 1,25D_3 also inhibits the *in vitro* and *in vivo* growth of the murine SCC cell line SCCVII/SF (6); antiproliferative activity in this model is associated with G_0-G_1 cell cycle arrest (7, 8) and induction of apoptosis (9). 1,25D_3 and 1,25D_3 analogues have been studied in combination with several cytotoxic drugs including platinum-containing agents (7, 10, 11), taxanes (12–14), and DNA-intercalating agents (13, 15). Such combinations display antitumor activity greater than that observed for any single agent. The combination of 1,25D_3 with cisplatin demonstrates increased activity against breast (11), prostate (10), and SCC cancer cell lines (7). Optimal cytotoxicity for the combination in SCC requires pretreatment of cells with 1,25D_3 for at least 24 h before treatment with cisplatin (7). The molecular mechanism by which antitumor activity is enhanced for this combination is unknown.

Cisplatin cytotoxicity results from the formation of bifunctional, intrastrand DNA adducts (16). In eukaryotic cells, DNA damage results in stabilization and activation of the tumor suppressor p53 (17). Such activation results in increased transcription of p53 target genes including the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ (18), the proapoptotic Bcl-2 family member *Bax* (19), and other genes implicated in cell cycle arrest, DNA repair, and apoptosis (20). p53 signaling plays a role in dictating the outcome of cisplatin

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[2] P. A. H. and T. F. M. contributed equally to this work.
[3] To whom requests for reprints should be addressed, at University of Pittsburgh Cancer Institute, W1003 Biomedical Science Tower, 200 Lothrop Street, Pittsburgh, PA 15213. Phone: (412) 624-0379; Fax: (412) 624-7737; E-mail: hershbergerpa@msx.upmc.edu.

[4] The abbreviations used are: 1,25D_3, 1,25-dihydroxyvitamin D_3; SCC, squamous cell carcinoma; Erk, extracellular signal-regulated kinase; MEK, Erk kinase; MAPK, mitogen-activated protein kinase; MEKK-1, MAPK kinase kinase; JNK, c-Jun NH_2-terminal kinase; PARP, poly(ADP) ribose polymerase; PCNA, proliferating cell nuclear antigen; EtOH, ethanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CI, combination index.

exposure; modulation of the expression of p53 or p53 target genes alters cellular sensitivity to cisplatin (21–26). In addition, cisplatin cytotoxicity may be attenuated by factors that regulate the intracellular accumulation of platinum, DNA repair, and tolerance to DNA damage (27).

Stress and survival signaling molecules also play a critical role in determining sensitivity to cytotoxic agents such as cisplatin. For example, in certain cancer cell lines, genotoxic agents activate MEK-Erk signaling, resulting in decreased cytotoxicity of those agents (reviewed in Ref. 28). Ovarian carcinoma cell lines demonstrate Erk activation and reduced cytotoxicity upon exposure to cisplatin, and treatment of these cells with the MEK inhibitor PD98059 increases cisplatin cytotoxicity (29, 30). Several reports have also demonstrated an important role for stress signaling molecules such as MEKK-1 in regulating cisplatin sensitivity. MEKK-1 becomes phosphorylated and is subsequently cleaved by caspase-3 upon treatment with cisplatin (31). Cleavage leads to the loss of intact MEKK-1 and the generation of a 91-kDa constitutively active COOH-terminal MEKK-1 catalytic domain that is released into the cytoplasm (31). Aberrant expression of unregulated MEKK-1 activity in the cytoplasm leads to the generation of proapoptotic signals via the c-Jun N-terminal kinase kinase (SEK1)-JNK-Jun and MKK3/6-p38 MAPK stress pathways and ultimately leads to apoptosis (32–34).

Recently, we reported that the proapoptotic effects of 1,25D_3 in SCC are associated with up-regulation of MEKK-1 before the onset of apoptosis (9). In addition, 1,25D_3 induces caspase-dependent proteolytic cleavage of MEK with consequent inhibition of MEK-Erk mitogenic/survival signaling. 1,25D_3 also decreases the level of phosphorylated/activated c-Jun, and either maintains or induces phospho-p38 MAPK (9). Based on these findings, we proposed that the loss of MEK/Erk survival signaling and the increase in MEKK-1/p38 MAPK stress signaling shift the balance from cell survival toward cell death in response to 1,25D_3 treatment. Interestingly, MEK cleavage and MEKK-1 up-regulation appear to be selective markers for apoptosis induced by 1,25D_3 [*i.e.,* they are either not observed or observed to a lesser extent for apoptosis induced by traditional cytotoxic agents, including cisplatin (9)]. In fact, in SCC cells, 1,25D_3 and cisplatin display opposite effects on the levels of intact MEKK-1 and phospho-Jun (9).

We have focused on alterations in cellular platinum, formation of platinum-DNA adducts, and effects on known components of cisplatin and 1,25D_3 signaling pathways in an attempt to elucidate the molecular mechanism of enhanced antitumor activity for the combination of 1,25D_3 plus cisplatin. Here, we report that SCC cells treated with the combination of 1,25D_3 and cisplatin exhibit an apoptotic response that is characteristic of 1,25D_3 and that the up-regulation of MEKK-1 observed for 1,25D_3 alone is potentiated by cotreatment with cisplatin. These data suggest that 1,25D_3 pretreatment commits cells to undergo apoptosis using a specific molecular pathway and that traverse along this pathway is increased when cells receive an additional, low-level genotoxic stimulus.

Materials and Methods

Chemicals and Reagents. 1,25D_3 (Hoffmann-LaRoche, Nutley, NJ) was reconstituted in 100% EtOH and stored, protected from light, under nitrogen at −70°C. All handling of 1,25D_3 was performed with indirect lighting. 1,25D_3 was diluted in tissue culture medium just before use. Cisplatin (Platinol-AQ; Bristol Laboratories, Princeton, NJ) was obtained as a 1 mg/ml solution and diluted in sterile saline or tissue culture medium immediately before use.

SCC Cells. Murine SCC cells (SCCVII/SF) were obtained and passaged as described previously (6). Cells were plated in RPMI 1640 supplemented with 12% fetal bovine serum (HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin sulfate and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were allowed to attach overnight and then treated and analyzed as described below. EtOH, used as a solvent control, never exceeded a final concentration of 0.0004%.

MTT Assay and Dose-Effect Analysis. SCC cells (1.5×10^4 cells/ml) were seeded in a volume of 0.1 ml into 96-well tissue culture plates. Cells were pretreated for 24 h by adding medium alone or medium containing 1,25D_3 directly to the wells. Cells were either incubated without further treatment or treated with medium containing cisplatin for an additional 24 h. The final volume in each well was 0.2 ml. To quantitate cell viability, the cells were incubated with 20 μl of 0.5% MTT for 90 min at 37°C. The medium was removed, and the cells were solubilized for 20 min in 10% SDS/10 mM HCl. Absorbance was read with an ELISA plate reader (Bio-Tek Instruments, Winooski, VT) at a wavelength of 490 nm. Median doses for each drug were determined from the dose-response data using CalcuSyn software (Biosoft, Ferguson, MO). Drug interactions were quantitated using the equation $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$. $(D)_1$ and $(D)_2$ are the doses of drug 1 and drug 2 that, when given in combination, inhibit cell growth by a specified percentage. $(Dx)_1$ and $(Dx)_2$ are the doses of drug 1 and drug 2 that, when given individually, inhibit cell growth by the same percentage. CI values of <1, 1, and >1 indicate synergism, additivity, and antagonism between the drugs, respectively.

Determination of Total Cellular Platinum. SCC cells (2.5×10^6 cells/T75 flask) were treated with either EtOH or 10 nM 1,25D_3. After 24 h, the medium was replaced with fresh medium containing cisplatin (0.5 μg/ml), and cells were incubated for an additional 0.5–2.0 h at 37°C. At the conclusion of the incubation, the tissue culture flasks were placed on ice, and the cells were harvested by rapid mixing with glass beads. The cells were collected by centrifugation (5 min at 200 × *g* at 4°C) and washed in ice-cold PBS. The resulting cell pellets were resuspended in ice-cold PBS, and an aliquot of each cell suspension was removed for cell count determination. The remaining cells were transferred to an Eppendorf tube and collected by centrifugation. The final cell pellet was dissolved in 0.25% Triton X-100, and total cellular platinum was determined by flameless atomic absorption spectroscopy using a Perkin-Elmer model 1100 spectrometer (Perkin-Elmer, Norwalk, CT) monitoring 265.9 nm, as described previously (35). Platinum concentration

was determined by comparison with a standard curve and was expressed on a per cell basis.

Determination of Platinum-DNA Adducts. Triplicate sets of three T75 flasks each of SCC cells (1.25×10^6 cells/flask) were pretreated with either EtOH or 10 nM $1,25D_3$ for 24 h. Cisplatin was added directly to pretreated cultures to a final concentration of 0.5 μg/ml, and cells were incubated for an additional 2 h. The tissue culture medium was removed, and adherent cells were recovered by trypsinization. DNA extraction was initiated by pooling cells from three identically treated flasks into a single tube. Cells were collected by centrifugation and washed twice with PBS. Cell pellets were lysed in a buffer containing 10 mM Tris-Cl (pH 8.0), 0.1 M NaCl, 0.1 mM EDTA, 0.5% SDS, and 20 μg/ml RNase and incubated for 5 h at 37°C. Proteinase K was then added to a final concentration of 100 μg/ml, and the preparation was incubated overnight at 50°C. DNA was extracted with phenol-chloroform and precipitated with EtOH. DNA precipitates were suspended in distilled H_2O, and the DNA yield was determined by reading the absorbance at 260 nm. Platinum-DNA adduct levels were quantitated using a ^{32}P postlabeling assay (36) and are expressed as fmol adducts/μg DNA.

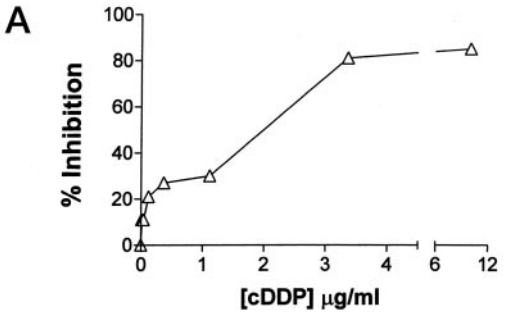
Preparation of Cell Lysates and Western Blotting. SCC cells (either 2×10^5 or 4×10^5 cells/T75 flask) were pretreated for 24 h with EtOH or 10 nM $1,25D_3$. Cells were incubated without further treatment, or cisplatin was added directly to tissue culture medium to a final concentration of 0.5 or 5.0 μg/ml. Cells were incubated for an additional 24 h. For caspase-3 Western blots only, adherent cells were scraped up into the medium containing floating cells. For all other analyses, medium in the flasks was swirled and removed to collect floating cells. Attached cells were then scraped off the flasks into PBS and placed in a separate tube. Protein extracts were obtained by lysing the pooled, attached, or floating cell populations in Triton X-100/SDS buffer as described previously (9). Equivalent amounts of protein from each sample were electrophoresed under denaturing conditions on SDS-polyacrylamide gels. Membranes were blocked and probed as described previously (8, 9). The antibodies used were anti-caspase 3 (210-807-C100; Alexis Corp., San Diego, CA), anti-PARP (C2.10; Enzyme Systems Products, Livermore, CA), anti-p53 (sc-99; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bax (13686E; PharMingen, San Diego, CA), anti-$p21^{Cip1}$ (13436E; PharMingen), anti-phospho-MEK1/2 (9121S; Cell Signaling Technologies, Beverly, MA), anti-phospho-Erk1/2 (sc-7383; Santa Cruz Biotechnology), anti-phospho-c-Jun (sc-822; Santa Cruz Biotechnology), and anti-MEKK-1 (sc-252; Santa Cruz Biotechnology). Secondary antimouse and antirabbit horseradish peroxidase-conjugated antibodies were from Amersham Biosciences (Piscataway, NJ). Actin was detected using the actin (Ab-1) kit from Oncogene Research Products (Boston, MA). Caspase-3 antibody reactions were visualized using SuperSignal substrate (Pierce, Rockford, IL). All other reactions were visualized using Renaissance Enhanced Chemiluminescence Reagents (NEN Life Science Products, Boston, MA).

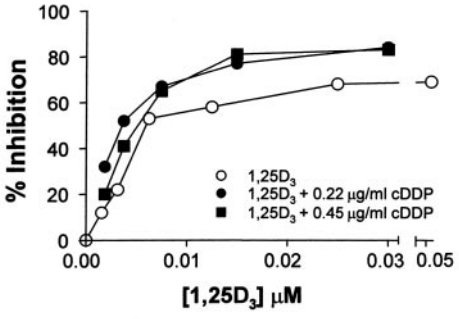
Results

Growth Inhibition and Induction of Apoptosis in SCC by the Combination of $1,25D_3$ and Cisplatin. To explore the nature of the interaction between $1,25D_3$ and cisplatin in SCC, cells were treated with varying concentrations of either $1,25D_3$ or cisplatin alone, or they were pretreated with $1,25D_3$ followed by cisplatin ($1,25D_3$/cisplatin); antiproliferative effects were measured using the MTT assay. As shown in Fig. 1*A,* greater inhibition of cell growth was observed for the combination than for either single agent. From these dose-effect data, it was determined that the concentrations of $1,25D_3$ and cisplatin required for 50% growth inhibition were 3–8-fold lower, respectively, when the drugs were used in combination than when used individually (Fig. 1*A*). These data were also used to determine CI values as described in "Materials and Methods." For most of the dose combinations tested, a CI value of <1 was obtained, indicating that the interaction between $1,25D_3$ and cisplatin is synergistic (Fig. 1*B*). Median dose-effect analysis also revealed apparent antagonism between the drugs when low concentrations of $1,25D_3$ were used in combination with higher cisplatin concentrations. However, no such antagonism is observed using *in vitro* clonogenic, *in vivo* excision clonogenic, or tumor outgrowth assays (7).

To dissect the mechanism for the enhanced antitumor effect of $1,25D_3$/cisplatin therapy, a single dose of each agent was selected for combination, and the effects on induction of apoptosis were determined by monitoring caspase-3 cleavage. SCC cells were pretreated with either vehicle (EtOH) or 10 nM $1,25D_3$. At this concentration, $1,25D_3$ treatment results in 50% growth inhibition (Fig. 1*A*). Cisplatin was added directly to pretreated cultures to a final concentration of 0.5 μg/ml. This concentration of cisplatin was anticipated to interact favorably with $1,25D_3$, based on the results presented in Fig. 1. $1,25D_3$ induced apoptosis, as evidenced by the accumulation of caspase-3 cleavage fragments (Fig. 2). This agrees with our previous data demonstrating cleavage of the caspase-3 substrate, PARP, in $1,25D_3$-treated SCC (9). Caspase-3 cleavage was not detected in cells treated with cisplatin alone (Fig. 2). However, in cells treated with $1,25D_3$/cisplatin, caspase-3 cleavage was consistently enhanced above that observed for cells treated with $1,25D_3$ alone (Fig. 2, *A* and *B*). These data suggest that the increase in antiproliferative activity observed for the combination of $1,25D_3$ plus cisplatin is attributed, at least in part, to an increase in apoptosis.

Effects of $1,25D_3$ Pretreatment on the Cellular Accumulation of Platinum and Formation of Platinum-DNA Adducts. To explore whether $1,25D_3$ increases cisplatin toxicity in SCC by increasing either cellular platinum accumulation or the formation of DNA adducts, SCC cells were pretreated for 24 h with vehicle or $1,25D_3$ and then treated with 0.5 μg/ml cisplatin for specified times. These times were selected based on observations that a 1-h exposure to cisplatin was sufficient to observe an increase in DNA platination in HL60 cells induced to differentiate with $1,25D_3$ (37). Cells were harvested, and we measured either cellular platinum concentration (Table 1) or platinum-DNA adducts (Table 2). Cellular platinum concentrations were not statistically





	Single agent IC50	*Combination IC50*
1,25D_3	0.012 μM	0.004 μM
cDDP	0.970 μg/ml	0.115 μg/ml

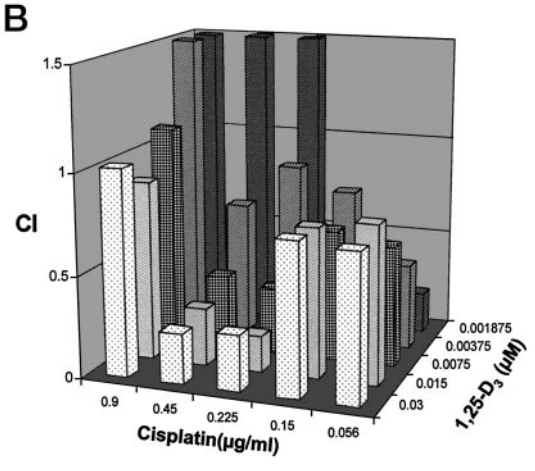
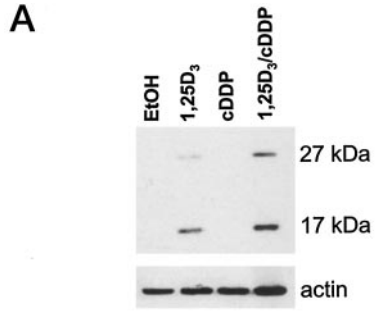


Fig. 1. The interaction between 1,25D_3 and cisplatin is synergistic in SCC. SCC cells were pretreated for 24 h with either media alone or the specified concentrations of 1,25D_3. Cells were incubated without further treatment or treated with the indicated concentrations of cisplatin (cDDP) for an additional 24 h. Plates were harvested by staining with 0.5% MTT, and the absorbance was read at 490 nm. *A,* dose-response curves for cisplatin alone (△), 1,25D_3 alone (○), or selected combinations (● and ■) are shown. Dose-effect data were used to calculate IC_{50} values. *B,* CI values were calculated as described in "Materials and Methods."

different in cells treated with 1,25D_3/cisplatin as compared with cells treated with cisplatin alone at any of the time points examined. Similarly, platinum-GG adducts, the major cross-linked species in cisplatin-treated cells (38), and platinum-AG adducts were not statistically different in cells treated with cisplatin alone compared with cells treated with 1,25D_3/cisplatin.



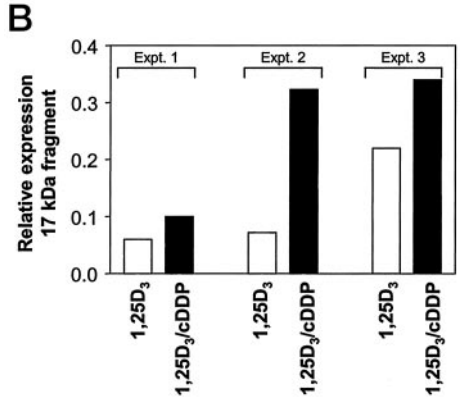


Fig. 2. Treatment of SCC with 1,25D_3/cisplatin increases apoptosis above that observed for either agent alone. SCC cells were pretreated with either EtOH or 1,25D_3 for 24 h. Cells were incubated without further treatment or treated by adding cisplatin directly to the tissue culture medium. Cells were harvested after an additional 24 h of incubation. *A,* protein (20 μg) extracted from pooled adherent and floating cells was resolved by SDS-gel electrophoresis and analyzed by Western blot using an antibody that detects cleaved caspase-3. The expected size for the active caspase-3 cleavage fragment is 17 kDa. The band migrating at approximately 27 kDa likely represents a caspase-3 cleavage fragment generated by calpain. To control for loading, the caspase-3 blot was reprobed for actin. *B,* densitometry was used to quantitate Western blot data from three experiments. The blot shown in *A* is from experiment 3. Relative expression values were obtained by normalizing the values for the 17-kDa caspase-3 cleavage fragment to the same samples' actin.

Effects of 1,25D_3 Pretreatment on Cisplatin Signaling Pathways in SCC. We subsequently tested the hypothesis that the increased apoptosis observed in SCC cultures treated with 1,25D_3/cisplatin results from a 1,25D_3-mediated increase in cisplatin signaling. After treatment with 1,25D_3, cisplatin, or 1,25D_3/cisplatin, two distinct populations of cells were evident; one adherent and the other floating (no floating cells were present in control cultures). Our prior work with 1,25D_3 demonstrated different molecular changes in these two populations (9); therefore, they were harvested and analyzed separately. Because the concentration of cisplatin used in these studies (0.5 μg/ml) did not induce caspase-3 activation (Fig. 2), in some experiments, cells were also treated with a 10-fold higher concentration of cisplatin as a positive control for cisplatin-induced apoptosis. Whole cell extracts were prepared, and immunoblot analysis was carried out for p53, p21, and Bax (to monitor p53 signaling) and phospho-c-Jun (to monitor JNK activation). Cleavage of PARP was also assessed so that molecular changes could be correlated with induction of programmed death.

Table 1 Effects of $1,25D_3$ pretreatment on platinum accumulation in SCC

SCC cells were pretreated for 24 h with EtOH or 10 nM $1,25D_3$. The media were replaced with fresh media containing cisplatin (0.5 μg/ml), and cells were incubated for an additional 0.5–2.0 h at 37°C. At the time points indicated, cells were harvested, and cellular platinum concentration was determined by flameless atomic absorption spectroscopy as described in "Materials and Methods." The data shown are the mean platinum concentration/cell (± SE) for two to five determinations per group. No statistically significant differences were observed in total platinum concentration in cells pretreated with EtOH *versus* $1,25D_3$ ($P = 0.26$ at 0.5 h, 0.69 at 1.0 h, and 0.90 at 2.0 h; Student's *t* test).

Pretreatment	Platinum concentration (fg/cell)		
	0.5 h	1.0 h	2.0 h
EtOH	59.5 ± 2.4	59.9 ± 8.6	85.6 ± 21.4
$1,25D_3$	68.9 ± 5.8	65.8 ± 10.7	82.4 ± 11.5

Table 2 Effects of $1,25D_3$ pretreatment on formation of platinum-DNA adducts in SCC

SCC cells were pretreated with either EtOH or 10 nM $1,25D_3$ for 24 h. Cisplatin was added directly to pretreated cultures to a final concentration of 0.5 μg/ml, and cells were incubated for an additional 2 h. Genomic DNA was extracted, and platinum-DNA adducts were quantitated as described in "Materials and Methods." Results are the mean ± SE for triplicate samples from a single experiment. No statistically significant differences in DNA adduct levels were observed between cells pretreated with EtOH *versus* $1,25D_3$ (GG adduct, $P = 0.73$; AG adduct, $P = 0.16$; Student's *t* test).

Pretreatment	Adducts (fmol/μg DNA)	
	GG	AG
EtOH	4.418 ± 0.108	0.535 ± 0.028
$1,25D_3$	3.590 ± 0.326	0.415 ± 0.063

Treatment with 0.5 μg/ml cisplatin modestly increased levels of full-length PARP in adherent cells and had little affect on PARP levels in floating cells (Fig. 3*A*). Based on the lack of PARP and caspase-3 cleavage (Figs. 2*A* and 3*A*) and the absence of membrane blebbing (data not shown), we conclude that this concentration of cisplatin, although promoting rounding and detachment of cells, did not induce apoptosis. However, SCC cells possess the molecular machinery to undergo cisplatin-induced apoptosis because treatment with 5.0 μg/ml cisplatin induced partial PARP cleavage in adherent cells and complete PARP cleavage in floating cells (Fig. 3*A*). $1,25D_3$ alone induced a significant decrease in PARP expression in the attached cells and a complete loss in the floating cells (Fig. 3*A*). In addition, $1,25D_3$ induced pronounced membrane blebbing in the floating cell population. Thus, in contrast to 0.5 μg/ml cisplatin, the population of floating cells in $1,25D_3$-treated cultures is apoptotic. This is in agreement with our previous findings (9). When $1,25D_3$ and 0.5 μg/ml cisplatin were combined, modulation of PARP levels resembled the effect of treatment with $1,25D_3$ alone. However, when $1,25D_3$ and 5.0 μg/ml cisplatin were combined, modulation of PARP levels was more similar to the effect of high-dose cisplatin alone (Fig. 3*A*).

We next assessed the expression of p53 and its downstream transcriptional targets, $p21^{Waf1/Cip1}$ and Bax, under the various treatment conditions. Treatment of cells with cisplatin (0.5 μg/ml) alone weakly induced p53 and its targets in both cell populations (Fig. 3, *A* and *B*). p53 induction was dose dependent, with much stronger induction observed at high (5.0 μg/ml) cisplatin concentrations (Fig. 3*A*). In contrast, $1,25D_3$ reduced p53, p21, and Bax expression in the adherent cells and p53 and p21 expression in the floating cells (Fig. 3, *A* and *B*). It is intriguing to note that although Bax (21 kDa) was reduced in adherent cells after $1,25D_3$ treatment, it was maintained at control levels in floating cells (Fig. 3*B* and discussed below). In addition, an 18-kDa Bax cleavage fragment was also detected in the floating cells (data not shown). Analogous to their differential effects on p53 expression, cisplatin and $1,25D_3$ treatment also led to differential effects on the stress signaling kinase, JNK. Whereas cisplatin treatment resulted in elevated c-Jun phosphorylation, phosphorylation was decreased to below control levels after $1,25D_3$ treatment (Fig. 3*B*). For each of the proteins examined, the molecular profile observed for cells treated with $1,25D_3$/cisplatin (0.5 μg/ml) resembled that of cells treated with $1,25D_3$ alone, whereas the profile observed for cells treated with $1,25D_3$/cisplatin (5.0 μg/ml) resembled that of cells treated with cisplatin (5.0 μg/ml) alone (Fig. 3, *A* and *B*).

Effects of Cisplatin on $1,25D_3$-induced Inhibition of Survival Signaling Molecules and MEKK-1 Up-Regulation. The inhibitory effect of $1,25D_3$ on cisplatin signaling makes it unlikely that the increase in antitumor activity for $1,25D_3$/cisplatin is due to $1,25D_3$ enhancement of cisplatin cytotoxicity. We therefore explored the alternative hypothesis that cisplatin enhances $1,25D_3$ proapoptotic signaling in SCC cells. In accord with our previous report (9), the apoptotic, floating cell population from $1,25D_3$-treated cultures demonstrated strongly reduced levels of MEK expression associated with the appearance of a 33-kDa cleavage fragment (Fig. 4*A*). As anticipated, the $1,25D_3$-mediated reduction in MEK resulted in reduced phosphorylation of Erk1/2, its downstream target (Fig. 4*A*; Ref. 9). In contrast, cisplatin treatment resulted in increased Erk phosphorylation/activation in the adherent cells (although MEK expression did not change; Fig. 4*A*). In general, the MEK/Erk profile in $1,25D_3$/cisplatin-treated cells resembled that of cells treated with $1,25D_3$ alone, except that the 33-kDa MEK cleavage fragment was further increased in the floating cells after treatment with $1,25D_3$/cisplatin as compared with cells treated with $1,25D_3$ alone (Fig. 4*A*).

When administered as a single agent, cisplatin (0.5 μg/ml) caused little or no increase in MEKK-1 expression in the adherent cell population and reduced expression in the floating cells (Fig. 4*B*). As we reported previously, $1,25D_3$ treatment was associated with up-regulation of the proapoptotic stress signaling kinase MEKK-1 in both adherent and floating cells (Fig. 4*B*; Ref. 9). MEKK-1 expression was increased to a similar extent in the cells that remained adherent after treatment with $1,25D_3$ alone and $1,25D_3$/cisplatin (Fig. 4*B*). After treatment with $1,25D_3$/cisplatin, a further increase in MEKK-1 expression was observed in the floating, apoptotic cells, which exceeded that observed for $1,25D_3$ alone (Fig. 4*B*). These data suggest that cisplatin enhances $1,25D_3$ proapoptotic signaling through MEKK-1.

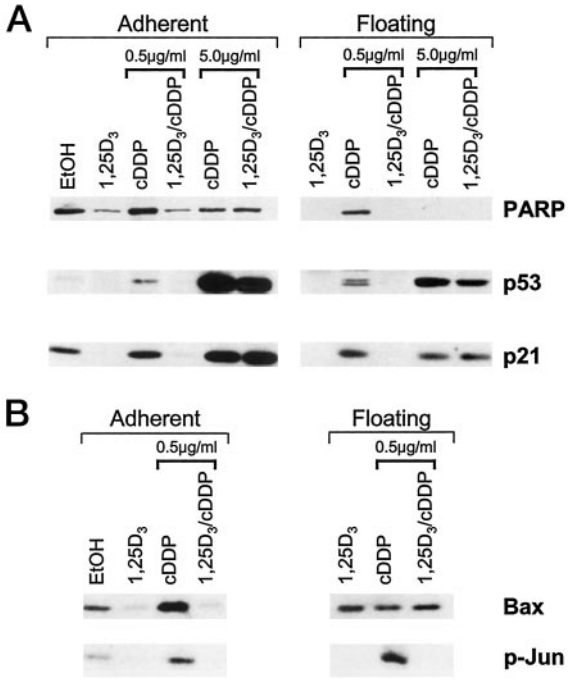


Fig. 3. 1,25D$_3$ and cisplatin differentially regulate levels of p53, p21, Bax, and phospho-Jun in SCC. SCC cells were pretreated with either EtOH or 10 nM 1,25D$_3$ for 24 h. Cells were incubated without further treatment or treated by adding cisplatin directly to the tissue culture medium to a final concentration of 0.5 or 5.0 μg/ml. Cells were harvested after an additional 24 h of incubation. Adherent and floating cells were harvested and analyzed separately. No floating cells were obtained from control (EtOH) cultures. Fixed amounts of protein were resolved by SDS-gel electrophoresis and analyzed by Western blot for PARP, p53, p21, Bax, or phospho-c-Jun. The blots shown in *A* and *B* were from separate experiments. For each protein analyzed, similar results were obtained in at least two additional experiments.

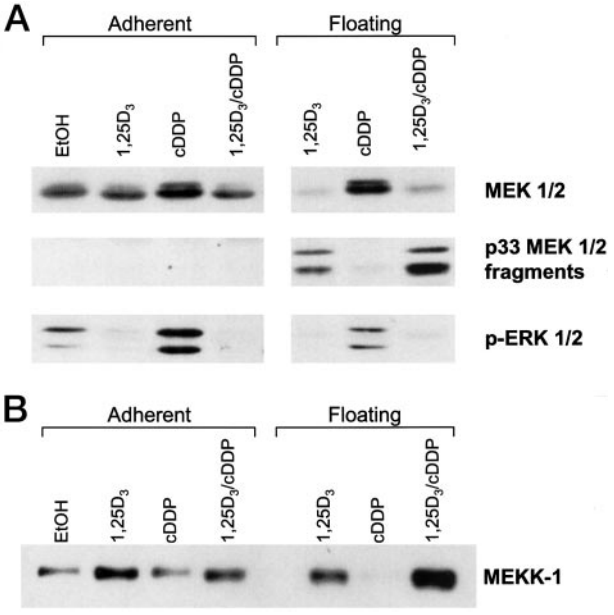


Fig. 4. MEKK-1 expression is increased by 1,25D$_3$ and increased further by treatment with 1,25D$_3$/cisplatin. SCC cells were treated and harvested as described in the Fig. 3 legend. No floating cells were obtained from control (EtOH) cultures. Fixed amounts of protein were resolved by SDS-gel electrophoresis and analyzed by Western blot for (*A*) MEK and phospho-Erk and (*B*) MEKK-1. For each protein analyzed, similar results were obtained in at least three additional experiments. It should be noted that cisplatin induction of phospho-Erk levels was variable between experiments.

Discussion

Antitumor activity is enhanced for combinations of 1,25D$_3$ (or 1,25D$_3$ analogues) with cytotoxic drugs including platinum-containing agents (7, 10, 11) and taxanes (12–14). The clinical implications of these laboratory findings are as follows: (*a*) for a fixed dose of cytotoxic drug, addition of 1,25D$_3$ may improve the antitumor response observed; and (*b*) a cytotoxic drug may be given at a reduced and potentially less toxic dose in combination with 1,25D$_3$ without loss of activity. As a step toward clinical development of 1,25D$_3$/cytotoxic combinations, Phase I clinical trials of 1,25D$_3$ plus carboplatin or paclitaxel are currently in progress at our institution (39). However, the mechanism by which antitumor activity is increased for such combinations is unclear. We report here that enhancement of antitumor activity for the combination of 1,25D$_3$ plus cisplatin in SCC is associated with increased apoptosis. By surveying known components of the 1,25D$_3$ and cisplatin signaling pathways, we find that the increase in apoptosis for the combination is associated with up-regulation of MEKK-1 to levels that exceed those observed for either agent alone. Our analysis of other molecular markers that are selectively induced by either 1,25D$_3$ or cisplatin reveals that the molecular profile of cells treated with 1,25D$_3$/cisplatin most closely resembles that of 1,25D$_3$. The mechanistic implication of these results is that cisplatin potentiates 1,25D$_3$ proapoptotic activity in SCC.

The ability of MEKK-1 to stimulate apoptosis is well documented (31, 34). These studies demonstrate that MEKK1-mediated apoptosis depends upon proteolytic processing at a caspase-3 cleavage site. Such processing results in a COOH-terminal fragment of MEKK-1 (ΔMEKK1) that stimulates apoptosis. We reported previously (9) that 1,25D$_3$ alone induces MEKK-1 up-regulation in both adherent and floating cell populations and that NH$_2$-terminal proteolytic processing of MEKK-1 (distinct from caspase-3 cleavage) occurs in those cells that become apoptotic after treatment. Ectopic overexpression of MEKK-1 has been reported to lead to accumulation of MEKK-1 in the cytosol and subsequent apoptosis (31). These findings have led to the concept that aberrant expression of MEKK-1 in the cytosol triggers the generation of apoptotic signals. Thus, if the strong up-modulation of MEKK-1 observed in 1,25D$_3$-treated cells results in its cytosolic expression, this may "prime" the cells to initiate an apoptotic signal upon the addition of a low-level cytotoxic stimulus. This hypothesis is supported by previous findings of Widmann *et al.* (40), who demonstrated that acute expression of ΔMEKK1 in 3T3 cells 17 h before cisplatin results in greater inhibition of cell number than treatment with cisplatin alone.

Based on our findings, we propose that the up-regulation of MEKK-1 resulting from 1,25D$_3$ pretreatment in SCC results in apoptotic sensitization, and this accounts for the

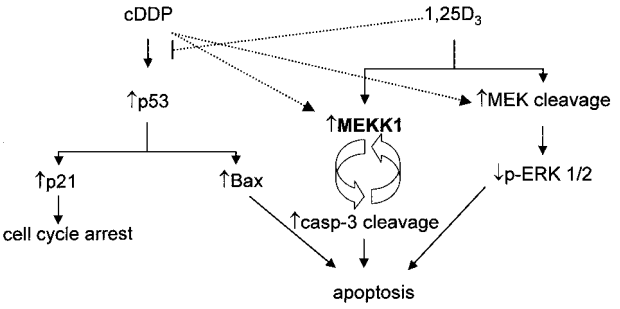


Fig. 5. Cisplatin enhances $1,25D_3$ proapoptotic signaling in SCC. $1,25D_3$ induces up-regulation of the stress signaling kinase MEKK-1 and promotes MEK cleavage, resulting in loss of MEK/ERK survival signals (Fig. 4; Ref. 9). MEKK-1 up-regulation occurs before the onset of apoptosis (9). In SCC, cisplatin induces p53 expression and increased expression of p53 targets, p21 and Bax. However, $1,25D_3$ pretreatment suppresses cisplatin induction of this response (Fig. 3). Addition of cisplatin to $1,25D_3$-pretreated cells results in further induction of MEKK-1 expression and further accumulation of MEK cleavage fragments (Fig. 4). Thus, cisplatin enhances signaling through the $1,25D_3$ proapoptotic pathway. We proposed previously that the increased expression of MEKK-1 (in the absence of MEK/ERK survival signals) promotes caspase-3 activation and apoptosis. Once activated, caspase-3 may mediate NH_2-terminal cleavage of MEKK-1, resulting in its further activation (31) and amplification of apoptotic signals. Molecular changes observed for each single agent are denoted by *solid arrows*; changes observed for the combination treatment are denoted by *dashed arrows*.

increase in apoptosis observed for the combination of $1,25D_3$ and cisplatin. However, it appears that cisplatin triggers a greater apoptotic response using a mechanism that is more characteristic of $1,25D_3$ than cisplatin. Thus, one possible interpretation of our data is that $1,25D_3$ pretreatment and consequent MEKK-1 up-regulation commit cells to a particular apoptotic pathway and that traverse along this pathway is enhanced in the presence of cisplatin (see Fig. 5). The signals downstream of MEKK-1 that lead to apoptosis remain largely undefined. However, one recent study demonstrates that conformational changes in the proapoptotic Bcl-2 family member Bak occur in response to MEKK-1 activation in cisplatin-treated melanoma cell lines (41). Future studies will be required to explore the exact biological consequences of $1,25D_3$-induced MEKK-1 up-regulation and its effects on Bak conformation in SCC.

The molecular survey done here indicates $1,25D_3$ modulates multiple factors that could increase cellular susceptibility to apoptosis. Of note in this regard are the changes we observe in the proapoptotic Bax protein, which promotes cell death by modulating mitochondrial release of proapoptotic factors such as cytochrome *c* (42). We find that there is an association between Bax expression and apoptosis induction in $1,25D_3$-treated SCC such that although Bax expression is reduced in viable cells that remain adherent, it is retained in the floating, apoptotic cells. In addition, Bax is cleaved to an 18-kDa species in the apoptotic cell population (data not shown). An 18-kDa calpain cleavage fragment of Bax has been identified (43) and reported to possess proapoptotic activity (44, 45), making it seem likely that this species also retains proapoptotic function in SCC cells. A role for $1,25D_3$-mediated alterations in Bax subcellular distribution and cleavage in the proapoptotic effects of $1,25D_3$ has been reported previously in MCF-7 breast cancer cells (46). It is interesting to note that the effects of $1,25D_3$ on Bax expression appear to be p53 independent because Bax is retained in floating cells with reduced p53 expression (Fig. 3, *A* and *B*).

We reported previously that $1,25D_3$-mediated apoptosis is associated with caspase-dependent cleavage of MEK, resulting in down-regulation of survival signaling through Erk (9). MEK cleavage, as evidenced by loss of full-length MEK and the appearance of a 33-kDa cleavage fragment, was consistently observed in the studies reported here (Fig. 4*A*). As compared with treatment with $1,25D_3$ alone, MEK cleavage fragments were further increased in cells that became apoptotic after treatment with $1,25D_3$/cisplatin. Cisplatin alone did not induce MEK cleavage or inhibition of phospho-Erk1/2 in the floating cells and did not induce apoptosis as evidenced by a lack of caspase-3 and PARP cleavage (Figs. 2*A* and 3*A*). Thus, these data indicate that cisplatin further potentiates the $1,25D_3$-mediated loss of survival signaling and are consistent with our hypothesis that cisplatin increases signaling through the $1,25D_3$ proapoptotic pathway.

Treatment with cisplatin alone has been reported to induce Erk phosphorylation/activation in ovarian cancer cell lines (47). In these cells, treatment with cisplatin in the presence of the MEK inhibitor PD98059 results in a reduction in cisplatin-induced p53 accumulation (47). These studies suggest Erk is one of the regulators of p53 protein accumulation during the DNA damage response to cisplatin. It is therefore tempting to speculate that the $1,25D_3$-mediated reduction in MEK/Erk signaling may, in part, account for the reduction in p53 observed in cells treated with $1,25D_3$ alone and the block to p53 induction observed in cells treated with $1,25D_3$ plus cisplatin (Fig. 3*A*). The inhibitory effect of $1,25D_3$ on p53 induction has been reported previously; pretreatment of MCF-7 cells with the $1,25D_3$ analogue EB1089 enhanced Adriamycin cytotoxicity and blocked Adriamycin induction of p53 (48).

$p21^{Waf1/Cip1}$ is transcriptionally up-regulated by p53 (18). Thus, $1,25D_3$-mediated inhibition of p53 may explain the suppression of p21 observed in $1,25D_3$-treated SCC cells (Fig. 3*A*). After DNA damage, p21 promotes cell cycle arrest by interacting with and inhibiting both cyclin-cyclin-dependent kinase complexes (49) and PCNA (50, 51). Although the interaction between p21 and PCNA results a block in DNA replication, this association may actually be required for efficient removal of DNA adducts via the nucleotide excision repair pathway. Consistent with this hypothesis, p21-deficient HCT116 colon cancer cells show a reduced capacity to repair cisplatin-damaged DNA, and this nucleotide excision repair deficiency is overcome by reintroduction of wild-type p21 but not p21 mutants lacking a PCNA-binding domain (52). More recent studies suggest that PCNA remains associated with DNA for prolonged periods in p21-deficient fibroblasts, which may ultimately result in reduced PCNA recycling and accumulation of unrepaired lesions (53). Although we did not observe a difference in DNA adduct formation after a 2-h exposure to cisplatin (Table 2), it is possible that $1,25D_3$, by reducing p21 expression, may inhibit the repair of cisplatin-induced DNA adducts at later time points and

thereby contribute to cytotoxicity. Studies to test this hypothesis are currently in progress.

Finally, we show that for most dose combinations tested, there is a synergistic interaction between 1,25D_3 and cisplatin in SCC (Fig. 1*B*). Similar observations have been reported for both breast and prostate cancer cells (10, 11). Our studies in SCC also reveal apparent antagonism between these agents, an effect that is restricted to combinations using very low concentrations of 1,25D_3 and concentrations of cisplatin ≥ 0.225 μg/ml. The short half-life of cisplatin in plasma as compared with tissue culture medium makes extrapolation of our *in vitro* cisplatin exposures to *in vivo* exposures difficult. However, we consistently find that the 1,25D_3/cisplatin combination exhibits greater antitumor activity than either single agent in *in vivo* excision clonogenic assays and tumor outgrowth studies (7).[5] These *in vivo* data predict enhanced rather than diminished clinical activity for this combination.

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