Calcitriol (1,25-Dihydroxycholecalciferol) Enhances Paclitaxel Antitumor Activity in Vitro and in Vivo and Accelerates Paclitaxel-induced Apoptosis

Pamela A. Hershberger, Wei-Dong Yu, Ruth A. Modzelewski, Robert M. Rueger, Candace S. Johnson, and Donald L. Trump

ABSTRACT

We demonstrated that calcitriol has antiproliferative activity in squamous cell carcinoma and prostatic adenocarcinoma and enhances the antitumor activity of platinum-based agents. In this study, we examined whether calcitriol also increases paclitaxel cytotoxicity. The effect of treatment on growth of the murine squamous cell carcinoma (SCCVII/SF) and human prostatic adenocarcinoma (PC-3) was determined by clonogenic assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and monitoring tumor growth. Treatment of SCC or PC-3 cells in vitro with calcitriol prior to paclitaxel significantly reduced clonogenic survival compared with either agent alone. Median-dose effect analysis revealed that calcitriol and paclitaxel interact synergistically. Treatment of SCC or PC-3 tumor-bearing mice with calcitriol prior to paclitaxel resulted in substantially greater growth inhibition than was achieved with either agent alone, supporting the combined use of calcitriol and paclitaxel in the treatment of solid tumors. To explore the molecular basis for the enhanced antitumor activity of this combination, the effect of treatment on p21Waf1 (p21), Bcl-2, and poly(ADP-ribose) polymerase expression was evaluated in PC-3. A 72-h pretreatment with calcitriol reduced p21 expression and increased paclitaxel cytotoxicity (measured after 24 h) without evidence of apoptosis [poly(ADP-ribose) polymerase cleavage]. After 48 h, paclitaxel-induced apoptosis, the extent of which was increased similarly by pretreatment or concurrent treatment with calcitriol. We therefore propose a model for calcitriol enhancement of paclitaxel cytotoxicity in which the “early” (24 h) effects are schedule dependent and not attributed to enhancement of paclitaxel-induced apoptosis. In contrast, the “delayed” (48-h) enhancement of paclitaxel activity by calcitriol is schedule independent and associated with acceleration of apoptosis.

INTRODUCTION

In addition to its classical role in bone and mineral metabolism, the secosteroid hormone, vitamin D₃ (calcitriol, 1,25-dihydroxycholecalciferol) has antiproliferative activity in solid tumor models both in vitro (1–7) and in vivo (1, 4, 7, 8). Calcitriol binding activates the vitamin D receptor, a member of the steroid nuclear receptor superfamily, resulting in modulation of the transcription of target genes (9). Calcitriol treatment induces expression of the cdk inhibitors, p21(Waf1/Cip1) (p21) and/or p27(Kip1) (p27), in breast (10), prostate (11), and pancreatic cancer cell lines (6) in vitro. These proteins block progression into S-phase by binding to and inhibiting cyclin/cdk complexes (12).

We determined that calcitriol inhibits growth of the murine SCC SCCVII/SF (2, 7) and the growth and metastatic potential of the Dunning rat prostatic adenocarcinoma, Mat-lylu (1). We subsequently evaluated the use of calcitriol in combination with cytotoxic agents and found that calcitriol synergistically enhanced the antitumor activity of cisplatin and carboplatin in vitro and in vivo, and that these effects were schedule dependent (13).

In SCC, mechanistic studies revealed that calcitriol induces Gₐ-G₁ arrest (13), a decrease in Rb phosphorylation, an increase in expression of p27, and a decrease in expression of p21 (14). Expression of p21 is also reduced in SCC tumors harvested from animals treated with therapeutic doses of calcitriol (14). These findings suggest that p21 down-modulation may be a component of the mechanism by which calcitriol exerts antiproliferative activity.

Recent studies indicate that a reduction in p21 expression sensitizes tumor cells to both DNA-damaging agents (15, 16) and microtubule-damaging agents such as paclitaxel (17–19). In MCF-7 breast carcinoma cells, paclitaxel induces p21 expression; treatment of these cells with antisense p21 oligonucleo-

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3 The abbreviations used are: cdk, cyclin-dependent kinase; SCC, squamous cell carcinoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; CI, combination index; PSA, prostate-specific antigen; PVDF, polyvinylidene difluoride.
tides increases paclitaxel cytotoxicity (17). Similarly, paclitaxel antitumor activity is increased in vitro and in vivo in HCT116 colon carcinoma cells made deficient in p21 expression (18). Compared with p21+/+ controls, these cells displayed an increase in paclitaxel-induced apoptosis.

Given the ability of calcitriol to decrease p21 expression in SCC in vitro and in vivo and the reported association between reduced expression of p21 and increased sensitivity to paclitaxel, we hypothesized that calcitriol would enhance the antitumor activity and apoptosis-promoting ability of paclitaxel.

MATERIALS AND METHODS

Tumor Cells and Model Systems. SCCVII/SF is a moderately well-differentiated SCC derived from a spontaneously arising tumor of the C3H mouse (20). SCCVII/SF cells were obtained from K. Fu (University of San Francisco, San Francisco, CA). They were transplanted in 6–10-week-old female C3H/HeJ mice (obtained from The Jackson Laboratory, Bar Harbor, ME). For in vitro studies, cells were grown in RPMI 1640 containing penicillin-streptomycin and 15% FCS (Hy-Clone Laboratories, Inc., Logan, UT) at 37°C in a humidified atmosphere containing 5% CO2. In vivo, SCCs were routinely produced by s.c. inoculation of 5 x 106 log-phase tissue culture cells in the right flank of each mouse. Studies were initiated 9 days later when tumors were palpable.

The human prostatic adenocarcinoma cell line PC-3 was obtained from the American Type Culture Collection (Manassas, VA). For in vitro studies, cells were grown in F-12K medium containing penicillin-streptomycin and 10% FCS and 2 mM L-glutamine at 37°C in a humidified atmosphere containing 5% CO2. In vivo, adenocarcinomas were routinely produced by s.c. inoculation of 2 x 106 log-phase tissue culture cells mixed 1:1 with Matrigel (Becton Dickinson, Bedford, MA) in the right flank of each nude mouse (Taconic Farms, Germantown, NY). Studies were initiated when tumors were palpable. Animals were used in accordance with Institutional Animal Care and Use Committee guidelines.

Chemicals and Reagents. Calcitriol (Hoffmann-LaRoche, Nutley, NJ) was reconstituted in 100% ethanol and stored protected from light under a layer of nitrogen gas at −70°C. All handling of calcitriol was performed with indirect lighting. Paclitaxel (Taxol; Bristol-Myers Squibb, Princeton, NJ) was purchased as a 6 mg/ml solution in Cremophor EL and was diluted in tissue culture medium or sterile saline just prior to use. The antibodies used in these studies were monoclonal mouse anti-PARP (Enzyme Systems, Livermore, CA), monoclonal mouse antihuman Bcl-2 (Dako, Carpinteria, CA), and polyclonal rabbit anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA). Antirabbit and antimouse horseradish peroxidase-conjugated secondary antibodies were purchased from American Life Sciences (Arlington Heights, IL) and Promega Corp. (Madison, WI), respectively. Actin was detected using the actin (Ab-1) kit from Oncogene Research Products (Boston, MA).

In Vitro Clonogenic Tumor Cell Survival Assay. Tumor cells were incubated for 24 h in T25 flasks (Corning Costar Corp., Cambridge, MA) with or without calcitriol. Cells were then either left untreated or treated with various concentrations of paclitaxel for an additional 24 h. The cells were then harvested and counted, and a fixed number of cells were replated into six-well tissue culture plates (Corning Costar). After a 7-day incubation at 37°C in a humidified atmosphere containing 5% CO2, cell monolayers were washed with saline, fixed with 100% methanol, and stained with 10% Giemsa. Colonies, defined as being >50 cells, were counted with the use of a light microscope. The surviving fraction is defined as follows: Surviving fraction = [fraction of viable cells recovered x (cloning efficiency of treated cells/cloning efficiency of untreated, control cells)].

MTT Assay and Drug Interaction Analysis. SCC cells were plated at 1.5 x 103 cells/well into 96-well tissue culture plates (Corning Glass Inc., Corning, NY) and incubated at 37°C in a humidified atmosphere containing 5% CO2. After 24 h, plates were harvested and stained with 0.5% MTT, and the absorbance was read with an ELISA reader (model EL-340; Bio-Tek Instruments, Winooski, VT) at 460 nm. Drug interactions were quantitated by median-dose effect analysis (21), and combination index values were derived using CalcuSyn software (Biosoft, Ferguson, MO), as described previously (22). CI values of <1, =1, and >1 indicate synergism, additivity, and antagonism between the drugs, respectively.

In Vivo Excision Clonogenic Assay. Mice with 9-day SCCs (three to five animals/group) were treated with saline or 2.5 μg of calcitriol each day for 3 days. On day 3, mice also received varying doses of paclitaxel. Twenty-four h after the last injection, the animals were sacrificed, and their tumors were excised. Aliquots of minced tumor were enzymatically dissociated for 60 min at room temperature with a mixture of type I collagenase, DNase, and EDTA. For each treatment group, a fixed number of viable tumor cells, as determined by trypan blue staining, were then plated in six-well tissue culture plates. After incubation for 7 days, colonies were counted, and the surviving fraction was calculated using the equation: Surviving fraction = (the cloning efficiency of treated cells/cloning efficiency of untreated, control cells). The surviving fraction per gram of tumor is defined as the number of clonogenic tumor cells per gram of treated tumor divided by the number of clonogenic tumor cells per gram of control, untreated tumor.

Tumor Growth Inhibition. To examine the in vivo antitumor activity of calcitriol, paclitaxel, or the combination of calcitriol with paclitaxel, treatment was initiated on animals bearing palpable SCC or PC-3 tumors. Animals were treated for 3 days with single, daily i.p. injections of saline or calcitriol. On day 3, animals also received a single i.p. injection of paclitaxel. Tumor measurements were obtained using calipers prior to initiating treatment (initial tumor volume) and on the days indicated. Tumor volumes were calculated by the following formula: volume = (length x width²)/2. For each tumor, fractional tumor volumes were calculated using the following formula: Fractional tumor volume = (volume on day measured)/(initial tumor volume).

Preparation of Cell Lysates and Western Blot Analysis. PC-3 cells were seeded into T75 flasks at densities of 1 x 10⁴ to 2 x 10⁴ cells/ml. Forty-eight h later, the medium was replaced. Treatments were done by adding concentrated drug
stocks directly to the culture media. At various times, cells that detached from the tissue culture plate were harvested by collecting the culture medium. After removing these cells, adherent cells were scraped up into PBS. Detached and adherent cells were maintained as separate populations. Cells were collected by centrifugation and washed once in PBS, and the resulting pellets were stored at −70°C. Protein extracts were prepared by resuspending cell pellets in lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris, and 150 mM NaCl, containing 1× protease inhibitor cocktail (PharMingen)] for 30 min on ice. Lysates were transferred to 1.5-ml Eppendorf tubes and clarified by centrifugation at 13,000 rpm for 10 min at 4°C. Proteins were quantitated in duplicate using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to manufacturer’s directions. Protein lysates were stored at −70°C until use.

Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions and then electrophoretically transferred to PVDF 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) and 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).

RESULTS
Calcitriol Increases Paclitaxel Antitumor Activity in Vitro. To examine the antitumor activity of calcitriol and paclitaxel alone, or in combination, murine SCC cells were used in an in vitro clonogenic assay. SCC cells were: (a) treated with paclitaxel or calcitriol alone; (b) pretreated for 24 h with calcitriol and then treated with paclitaxel; or (c) treated simultaneously with both agents. Paclitaxel was not administered prior to calcitriol in these studies because pilot in vivo experiments demonstrated that this schedule was associated with greater toxicity (data not shown). As we reported previously, calcitriol inhibits clonogenic survival in SCC with an IC_{50} of 4 nM (Fig. 1; Ref. 7). Paclitaxel alone also inhibits SCC survival, with an IC_{50} of 23 nM. Significantly greater antitumor activity was achieved when calcitriol was combined with paclitaxel (Fig. 1). Calcitriol pretreatment potentiated paclitaxel activity to a greater extent than concurrent treatment, except at the highest dose of paclitaxel studied. These results demonstrate that antitumor activity in SCC is increased by combining calcitriol with paclitaxel, and the optimal schedule for administration is treatment with calcitriol, followed by paclitaxel.

Similarly, we examined whether the combination of calcitriol and paclitaxel was effective in inhibiting the growth of human prostatic adenocarcinoma (PC-3) cells. PC-3 cells were treated in vitro for 24 h with or without calcitriol and received no further treatment or were treated for an additional 24 h with varying concentrations of paclitaxel. As shown in Fig. 2, calcitriol alone had detectable antiproliferative activity in these cells, with an IC_{50} of 5 μM. Paclitaxel, when used as a single agent, reduced PC-3 clonogenic survival in a concentration-dependent manner. Significantly greater growth inhibition was achieved by pretreating the cells with calcitriol at each of the paclitaxel concentrations tested. Therefore, the combination of calcitriol plus paclitaxel displays increased antiproliferative activity in murine SCC and human prostatic adenocarcinoma, indicating that the effects are not cell type specific.

The Interaction between Calcitriol and Paclitaxel Is Synergistic in Vitro. To evaluate whether the combination of calcitriol and paclitaxel is synergistic in vitro, dose-response curves were generated using an in vitro clonogenic assay in SCC cells treated with calcitriol alone (Δ), with varying doses of paclitaxel alone (○), pretreated with calcitriol followed by paclitaxel (●), or treated simultaneously with calcitriol plus paclitaxel (◇), as measured by growth inhibition in the 7-day in vitro clonogenic assay. As shown in Fig. 3, the combination of calcitriol plus paclitaxel resulted in a significantly greater decrease in surviving fraction compared with either agent alone, the excision clonogenic assay was used. As shown in Fig. 4A, the combination of calcitriol plus paclitaxel resulted in a significantly greater decrease in surviving fraction.
Calcitriol Enhances Paclitaxel Antitumor Activity

Fig. 2 Dose-response curves of PC-3 cells treated in vitro with calcitriol alone (▲), with varying doses of paclitaxel alone (○), or pretreated with calcitriol for 24 h followed by paclitaxel (●), as measured by growth inhibition in the 7-day in vitro clonogenic assay. Cells were exposed to paclitaxel for 24 h. Calcitriol was used at a concentration of 5 μM. Each point represents the mean surviving fraction as determined by counting triplicate wells; bars, 95% confidence intervals. Note that the symbol for the calcitriol-alone treatment group is arbitrarily placed with respect to the X axis. Values for pretreatment with calcitriol followed by paclitaxel are significantly different from those obtained for paclitaxel alone; *, P < 0.0004.

Fig. 3 Assessment of the interaction between calcitriol and paclitaxel in SCC. Cells were plated at 1.5 × 10⁶ cells/well into 96-well tissue culture plates and allowed to recover for 24 h. Cells were then either untreated or pretreated for 24 h with the indicated doses of calcitriol. Subsequently, cells received either no further treatment or were treated for 24 h with the indicated doses of paclitaxel. Plates were harvested by staining with MTT, and the absorbance was read at 460 nm. The dose-effect data obtained for each drug alone and in combination were used to calculate CI values as described previously (22).

as compared with paclitaxel or calcitriol alone. Thus, greater in vivo antitumor activity is achieved in SCC by pretreatment with calcitriol followed by paclitaxel.

To determine whether an increase in clonogenic cell kill was associated with inhibition of tumor growth, SCC tumor-bearing mice were treated with saline, calcitriol or paclitaxel alone, or calcitriol in combination with paclitaxel. We used a schedule of daily × 3 doses of calcitriol with paclitaxel administered on day 3. This calcitriol dosing regimen was reported previously to maximize antitumor efficacy while minimizing toxicity or hypercalcemia (13). In SCC, paclitaxel had no significant activity when used as a single-agent therapy, and calcitriol decreases p21 expression in SCC (14), we hypothesized that calcitriol enhances paclitaxel antitumor activity via its effects on p21. To test whether calcitriol treatment decreases p21 expression in PC-3 as it does in SCC, cells were treated in vitro with ethanol vehicle control or calcitriol. At various times, whole cell lysates were prepared and analyzed for p21 expression by Western blot. As shown in Fig. 6, calcitriol treatment resulted in a 60% decrease in p21 expression in PC-3 cells after 72 h and an 80% decrease in expression after 96 h.

To determine whether PC-3 cells with reduced p21 expression are more sensitive to paclitaxel than cells with baseline p21 expression, cells were pretreated for 72 h with ethanol vehicle control or calcitriol as indicated in Table 1. Cells subsequently received no further treatment or were treated with paclitaxel alone or calcitriol plus paclitaxel. Viable cells were counted 24 h after paclitaxel addition. Coadministration of calcitriol plus pa-
Fig. 4  Calcitriol increases paclitaxel antitumor activity in SCC in vivo.  
A, SCC tumor-bearing mice were treated with saline (C) or 2.5 μg of calcitriol each day for 3 days ( ). On the third day, mice also received varying i.p. doses of paclitaxel (0–60 mg/kg). Twenty-four h later, tumors were harvested, dissociated, and plated in the excision clonogenic assay. Colonies were enumerated after 7 days. Each point represents the mean surviving fraction for total clonogenic cells/gram of tumor (three to five mice per treatment group). *, values for treatment with calcitriol followed by paclitaxel are significantly different from those obtained for paclitaxel alone; P < 0.01. 
B, C3H mice bearing palpable, s.c. SCC tumors were treated with either saline (M), 1.25 mg of calcitriol daily for 3 days (Œ), 20 mg/kg paclitaxel on day 3 (E), or the combination of 1.25 mg of calcitriol daily for 3 days plus 20 mg/kg paclitaxel on day 3 (F). Both agents were administered i.p. Tumor measurements were obtained on the days indicated, and fractional tumor volumes were calculated as described in “Materials and Methods.” Data points represent the mean fractional tumor volume for five animals/group; bars, SD. Values significantly different from no treatment are shown. *, P < 0.01.

Fig. 5  Calcitriol increases paclitaxel antitumor activity in PC-3 in vivo.  
A, nude mice bearing palpable, s.c. PC-3 tumors were treated with either saline (☐), 0.75 μg of calcitriol daily for 3 days (▲), 10 mg/kg paclitaxel on day 3 (•), or the combination of 0.75 μg of calcitriol daily for 3 days plus 10 mg/kg paclitaxel on day 3 ( ●). Both agents were administered i.p. Fractional tumor volumes were calculated as described in the legend for Fig. 4B. Data points represent the mean fractional tumor volume for five animals/group; bars, SD. Arrows, treatment days. 
B, animals treated previously with calcitriol plus paclitaxel on days 8–10 were treated with a second cycle of therapy on days 29–31. m, the slope of the growth curve derived by linear regression of tumor volume data obtained on the days indicated.
Calcitriol Enhances Paclitaxel Antitumor Activity

Western blot using polyclonal anti-p21 antibodies. Protein expression levels were quantitated by densitometry and are expressed as a percentage of ethanol control at each time point. A representative experiment is shown.

FIG. 6 Calcitriol modulates expression of the cdk inhibitor p21 in PC-3 cells. Whole cell lysates were prepared from subconfluent PC-3 cells treated in vitro with ethanol solvent control or 5 μM calcitriol for 24–96 h. Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions, transferred to PVDF membrane, and analyzed by Western blot using polyclonal anti-p21 antibodies. Protein expression levels were quantitated by densitometry and are expressed as a percentage of ethanol control at each time point. A representative experiment is shown. EtOH, ethanol.

Paclitaxel resulted in a greater reduction in cell number than treatment with either agent alone (Table 1). However, the effect of the combination was only additive. In contrast, a greater than additive reduction in cell number was obtained when cells were pretreated with calcitriol followed by paclitaxel. Thus, a calcitriol pretreatment that reduces p21 expression further enhances paclitaxel cytotoxicity in PC-3.

Paclitaxel-induced apoptosis in PC-3 is associated with phosphorylation of Bcl-2 (23), which inactivates the apoptotic suppressor function of this protein (23–25). Therefore, to determine whether PC-3 cells with reduced p21 expression show enhanced paclitaxel activity at the molecular level, we examined whether calcitriol pretreatment increased or accelerated paclitaxel-induced changes in Bcl-2 expression and apoptosis. As outlined in Table 1, PC-3 cells were either treated with ethanol control or pretreated with 5 μM calcitriol for 72 h, a time sufficient for p21 down-modulation. Subsequently, cells received no further treatment, were treated with paclitaxel alone, or were treated with calcitriol plus paclitaxel for varying lengths of time. Inspection of treated cells revealed two morphologically distinct populations, one cell population remained adherent after treatment whereas a second population detached from the culture dishes. These populations were analyzed separately.

At 24 h, paclitaxel induced Bcl-2 phosphorylation in both the adherent and detached cell populations (Fig. 7). Whereas unphosphorylated Bcl-2 was most abundant in the paclitaxel-treated adherent cells, only the phosphorylated forms of Bcl-2 were detected in the detached cells. Phosphorylated Bcl-2 species were still detected in both cell populations after 48 h of treatment but were virtually absent by 72 h. Calcitriol had little effect on Bcl-2, and neither pretreatment nor concurrent treatment with calcitriol altered the effects of paclitaxel on Bcl-2 expression/phosphorylation.

Apoptosis, as measured by loss of full-length PARP, first became evident in the detached cells after 48 h of treatment (Fig. 7). At this time, paclitaxel treatment resulted in a 50% reduction in PARP. Although calcitriol itself did not induce apoptosis, it enhanced the effects of paclitaxel such that cells treated with calcitriol prior to or in combination with paclitaxel displayed a 78% reduction in PARP. By 72 h, PARP was no longer detected in the cells that detached after paclitaxel administration, and addition of calcitriol had no further discernible effect. Calcitriol did not alter the effects of paclitaxel on PARP expression in the adherent cell population at any of the times examined. The data obtained at 48 h indicate that calcitriol can accelerate paclitaxel-induced apoptosis in a subset of PC-3 cells in a schedule-independent manner.

DISCUSSION

On the basis of epidemiological findings, Schwartz and Hulka (26) proposed a protective role for calcitriol in prostate cancer. Subsequently, the antiproliferative activity of calcitriol on prostatic adenocarcinoma cell lines in vitro (1, 2, 11) and in vivo (1, 27) was demonstrated. Antiproliferative effects of calcitriol were also observed in a pilot clinical trial in which, in a small set of patients with early, recurrent prostate cancer, calcitriol decreased the rate of PSA rise, resulting in an increase in PSA doubling times (28). PSA responses have also been observed in our ongoing Phase II trial of calcitriol plus dexamethasone in hormone-refractory prostate cancer (29). In this trial, 8–12 μg of calcitriol was given p.o. Monday, Tuesday, and Wednesday each week with 4 mg of dexamethasone given Sunday, Monday, Tuesday, and Wednesday. Among evaluable patients, 21% experienced a greater than 50% decrease in PSA, and 79% experienced a decrease in PSA velocity. In a further effort to develop new calcitriol-based therapies for advanced malignancy, we investigated the effect of combining calcitriol with cytotoxic agents.

Preclinically, we demonstrated that there is an increase in antitumor activity in prostatic adenocarcinoma using calcitriol in combination with paclitaxel in vitro and in vivo as measured in clonogenic assays and tumor growth inhibition studies. On the basis of these findings, we propose that calcitriol plus paclitaxel combination therapy may have utility in the treatment of patients with prostate cancer.

The clinical use of calcitriol may be restricted by its dose-limiting toxicity, hypercalcemia. However, a variety of calcitriol analogues, including ILX-23–7553 and EB1089, have been described that possess antiproliferative activity in vivo without inducing hypercalcemia (7, 30). It has been shown recently that EB1089, when combined with paclitaxel, inhibits the growth of...
MCF-7 breast cancer cells in vivo (31). Furthermore, we observed that paclitaxel appears to attenuate calcitriol-mediated hypercalcemia in preclinical models (data not shown). Agents that disrupt or stabilize microtubules can inhibit calcium transport, which may account for this activity (32). Thus, paclitaxel and either calcitriol or analogues may be a safe and effective combination in the treatment of human cancer.

We have further demonstrated that calcitriol enhances paclitaxel antiproliferative activity in vitro and in vivo in the murine SCC model, SCCVII/SF. A previous report indicates that these cells are relatively resistant to paclitaxel in vivo at a concentration of 40 mg/kg (33). We found that although paclitaxel (20 mg/kg) has little activity when administered to tumor-bearing mice as a single agent, pretreatment with calcitriol yields substantial antitumor activity (4B). These data suggest that calcitriol and paclitaxel combination therapy may be useful, even in the treatment of tumors that are paclitaxel insensitive.

Paclitaxel cytotoxicity is increased in MCF-7 breast cancer cells and HCT116 colon cancer cells when p21 expression is specifically perturbed (17, 18). Because calcitriol treatment reduces p21 expression in the SCC model (14) and in PC-3 cells (Fig. 6), we hypothesized that calcitriol might enhance the antitumor activity of paclitaxel via its effect on p21. To test this, we examined whether paclitaxel effects were enhanced in cells pretreated with calcitriol (p21 low) as compared with cells receiving concurrent calcitriol (baseline p21) and paclitaxel. Paclitaxel effects were measured by following changes in viable cell number (Table 1) and molecular markers (Fig. 7).

### Table 1 Effect of in vitro treatment on recovery of viable PC3 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>First tx* (72 h)</th>
<th>Second tx (24 h)</th>
<th>Cell no. ($\times 10^6$)</th>
<th>% reduction (in cell no.)</th>
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<tr>
<td>Ethanol</td>
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</table>

*tx, treatment; Ptx, paclitaxel.

![Fig. 7 Paclitaxel modulates expression of apoptosis-associated proteins Bcl-2 and PARP in PC-3 cells.](image-url)

PC3 cells ($2 \times 10^5$) were seeded and allowed to recover for 48 h. Cells were then treated for 72 h with either ethanol solvent control or calcitriol, as indicated. For the second treatment, concentrated stocks of paclitaxel alone or calcitriol plus paclitaxel were added directly to the cultures. The final concentrations of calcitriol and paclitaxel were 5 μM and 100 nM, respectively. Twenty-four h after the second treatment, cells were harvested, and viable cells were counted using trypan blue. The percentage of reduction in viable cell number was calculated using the equation: 1 - (cell number treated /cell number EtOH control) × 100.
after paclitaxel addition. At this time, calcitriol alone does not induce apoptosis but does enhance the level of PARP cleavage observed in the detached cell population in the presence of paclitaxel. This enhancement is schedule independent, with similar effects observed in paclitaxel-treated cells regardless of whether they were pretreated or concurrently treated with calcitriol.

On the basis of these data, we propose a model in which the “early” (within 24 h) enhancement of paclitaxel cytotoxicity by calcitriol is schedule dependent and is not attributed to acceleration of paclitaxel-induced apoptosis. The schedule dependence may reflect the time required for calcitriol treatment to decrease p21 expression. The “delayed” (at 48 h) enhancement of paclitaxel activity by calcitriol is schedule independent and associated with acceleration of apoptosis in a subset of PC-3 cells. Recent work from our laboratory demonstrates that calcitriol inhibits specific survival signals in cells that detach during treatment. Such inhibition may render these cells more susceptible to the proapoptotic signals generated by paclitaxel. The ability of calcitriol to reduce cell survival signals may also explain how it can enhance the antitumor activity of mechanistically diverse cytotoxic agents, such as cisplatin (13) and paclitaxel. Studies to address these and related issues are in progress.

Paclitaxel-mediated apoptosis in LNCaP and PC-3 prostate cancer cells has been associated with Bcl-2 phosphorylation and inactivation (23) and/or down-modulation of the related apoptotic suppressor, Bcl-XL (34). Consistent with these results, we found that within 24 h, paclitaxel treatment resulted in phosphorylation of the apoptotic suppressor protein, Bcl-2. Loss or inactivation of Bcl-2 in prostate cancer cells after paclitaxel administration has been proposed to promote cell death by shifting the intracellular balance of death regulators in favor of proapoptotic molecules such as Bax (23). In our studies, paclitaxel-mediated changes in the intracellular levels of Bcl-2 temporally precede the loss of full-length PARP, suggesting that they may initiate the apoptotic program.

Wang et al. (35) demonstrated recently that calcitriol pretreatment increases paclitaxel induction of cell death and paclitaxel antitumor activity in vitro in MCF-7 breast cancer cells. However, in contrast to our findings, calcitriol modestly increased the effect of paclitaxel in Bcl-2 phosphorylation. Comparison of these two studies reveals that although a 24-h exposure to 100 nm paclitaxel results in minimal Bcl-2 phosphorylation in MCF-7, this exposure results in strong induction of Bcl-2 phosphorylation in PC-3. This strong induction may have prohibited the detection of a subtle effect of calcitriol on paclitaxel-mediated Bcl-2 phosphorylation in PC-3.

In summary, our data demonstrate that calcitriol enhances paclitaxel antitumor activity in PC-3 and SCC cells in vitro and in vivo and indicate that novel calcitriol/paclitaxel-based combination therapies may have significant clinical utility in the treatment of a variety of solid tumors.

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