Award Number: DAMD17-98-1-8549

TITLE: Modulation of Paclitaxel Antitumor Effects by Calcitriol: Preclinical Studies of Mechanism, Toxicity and Efficacy in Prostate Cancer

PRINCIPAL INVESTIGATOR: Donald Trump, M.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, Pennsylvania 15213

REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Modulation of Paclitaxel Antitumor Effects by Calcitriol: Preclinical Studies of Mechanism, Toxicity and Efficacy in Prostate Cancer

Donald Trump, M.D.

University of Pittsburgh
Pittsburgh, Pennsylvania 15213
e-mail: trumpdl@max.upmc.edu

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

1,25-dihydroxycholecalciferol calcitriol has antiproliferative activity in vitro and in vivo and enhances the antitumor activity of conventional cytotoxic agents such as cisplatin and carboplatin. In this study, we examined the antitumor effects of calcitriol in combination with paclitaxel in animal tumor model systems and in a Phase I clinical trial. Treatment of PC-3 and SCC cells in vitro with calcitriol 24h prior to paclitaxel reduced clonogenic potential compared to either agent alone. Treatment of tumor bearing animals with calcitriol prior to paclitaxel resulted in greater growth inhibition than was achieved with either agent alone. Paclitaxel induced apoptosis in PC-3 cells as evidenced by the time-dependent accumulation of cells with hypodiploid DNA content and the loss of full-length PARP. Paclitaxel effects were associated with the induction of p21 and loss of Bcl-2 expression. None of the measured molecular alterations induced by paclitaxel were further modulated by calcitriol. Calcitriol enhances the antitumor effects of paclitaxel in vitro and in vivo. In addition, paclitaxel and Zoledronate significantly decreased calcitriol mediated hypercalcemia in animal models. Clinically, patients with advanced tumors are being treated with weekly cycles of calcitriol (daily x3) and paclitaxel (day 3) and the trial continues to accrue patients.
FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

[Signature] 10/29/99

PI - Signature  Date
Table of Contents

(1) Front Cover .............................................................................................................. 1
(2) Report Documentation Page .................................................................................. 2
(3) Foreword .................................................................................................................. 3
(4) Table of Contents .................................................................................................. 4
(5) Introduction ............................................................................................................. 5, 6
(6) Body – Progress Report
    Methods .............................................................................................................. 7-13
(7) Key Research Accomplishments ........................................................................... 18
(8) Reportable Outcomes ........................................................................................... 19, 20
(9) Conclusions ............................................................................................................. 21-23
(10) References ............................................................................................................. 24-27
(11) Appendices:  Figures for Progress Report
                  Submitted Manuscript
                  Abstracts
Introduction

Cancer of the prostate is the most prevalent cancer of American men. At the time of diagnosis, almost 50% of patients have disease that extends beyond the prostate gland. Disseminated disease is primarily treated by local palliative measures and by testicular androgen ablation. More than 75% of asymptomatic patients benefit from hormonal manipulation. However, the median time to treatment failure after initiation of hormonal therapy is 18-24 months (7-10). Progression from androgen-dependent to androgen-independent prostate cancer (AIPC) occurs after hormonal therapy in almost all individuals. The median survival after onset AIPC is 10-12 months. New therapies for the treatment of androgen-independent prostate cancer are needed.

One approach for advanced disease is use of the seco-steroid hormone, calcitriol (1,25-dihydroxycholecalciferol). Epidemiologic data suggest that many of the major risk factors for clinically significant prostate cancer may be related to low serum levels of calcitriol (5). We have shown that calcitriol inhibits proliferation of the highly metastatic Mat-Ly-Lu (MLL) prostate cell line in vitro and in vivo and reduces the frequency of lung metastases in tumor-bearing animals (6). Calcitriol also inhibits the in vitro growth of other prostate cancer cell lines including ALVA-31, PC-3 and LNCaP (7,8).

The anti-proliferative effects of calcitriol appear to require the expression of the vitamin D receptor (VDR) (9,10). In general, greater VDR content is associated with a greater antiproliferative response (7). Calcitriol binding activates the VDR resulting in modulation of the transcription of target genes with promoters containing a vitamin D response element (VDRE). Among the proteins regulated by calcitriol in prostate cancer cell lines is p21Waf1/Cip1, the cyclin dependent kinase (cdk) inhibitor (11). We are evaluating the relationships between p21 modulation and other molecular changes and the interaction of calcitriol and paclitaxel, a potent microtubule stabilizing agent, currently used in the treatment of ovarian and breast carcinomas. In prostate cancer models paclitaxel inhibits both tumor cell proliferation and metastatic ability.
At nanomolar concentrations, paclitaxel induces apoptosis in these cells, which is associated with inactivation or down-modulation of the apoptotic suppressor proteins Bcl-2 and Bcl-X\textsubscript{L} (12,13). Paclitaxel alone has only limited activity in the treatment of AIPC in initial phase II clinical trials, but combinations with estramustine as well as prolonged treatment schedules appear to show greater activity.

We present here the progress we have made in studying the interactions of paclitaxel and calcitriol with the support of Department of Defense grant DAMD17-98-1-8549. We have chosen to evaluate combinations of paclitaxel + calcitriol in PC-3 (a human prostate cancer) and SCC (a murine squamous cell cancer) to investigate differences in response and biologic effects, hypothesizing that such differences may provide insight into the mechanism of interaction between these two agents.
Progress Report:

Statement of Work- Specific Aim 1: To determine the parameters for optimum potentiating activity in prostate model systems

Calcitriol pre-treatment increases paclitaxel activity in vitro in SCC and PC-3 cells.

To examine the antitumor activity of calcitriol and paclitaxel alone, or in combination, murine SCC cells were used in an in vitro clonogenic assay. These cells were selected for study initially since they are sensitive to calcitriol and are responsive only partially to paclitaxel. SCC cells were treated with paclitaxel or calcitriol alone, or were pre-treated with calcitriol then treated with paclitaxel, or treated simultaneously with both agents. Calcitriol has antitumor activity in SCC, and inhibits clonogenic survival 50% at a concentration of 4 nM (Fig. 1A). Paclitaxel alone also inhibits SCC survival, with 50% inhibition achieved at a concentration of 23 nM. Significantly greater antitumor activity was achieved when calcitriol was combined with paclitaxel; pre-treatment with calcitriol reduced the IC50 of paclitaxel to 0.03 nM in these cells, while simultaneous addition of calcitriol reduced the IC50 of paclitaxel to 2.3 nM. These studies demonstrate that antitumor activity in SCC is increased by combining calcitriol with paclitaxel, and that the optimal schedule for administration is pre-treatment with calcitriol followed by paclitaxel.

We next examined whether this combination was effective in inhibiting the growth of prostate cancer. PC-3 cells were treated for 24h with or without calcitriol and then received no further treatment or were treated for an additional 24h with varying concentrations of paclitaxel. Following treatment, the cells were re-plated at various dilutions and surviving colonies enumerated 7 days later. As shown in Fig. 1B, calcitriol alone had antiproliferative activity in these cells: IC50 concentration was approximately 5μM. Paclitaxel, when used as a single agent, reduced PC-3 clonogenic survival in a concentration-dependent manner. At each of the paclitaxel concentrations tested, significantly greater growth inhibition was achieved by pre-
treating the cells for 24h with calcitriol. Thus, the effect of combining calcitriol and paclitaxel is not cell type specific.

**In vivo effects of calcitriol and paclitaxel as measured in the excision clonogenic assay.**

To evaluate whether the combination of calcitriol and paclitaxel has greater *in vivo* antitumor activity than either agent alone, the excision clonogenic assay was used. Tumor-bearing C3H mice were treated for 3 days either with saline or 2.5µ g calcitriol each day. On the third day, mice were treated with varying intraperitoneal doses of paclitaxel (0 to 60 mg/kg). Twenty-four hours after paclitaxel administration, the tumors were harvested, dissociated, and plated in a clonogenic assay. As shown in Fig. 2, independent of the concentration of paclitaxel used, the combination of calcitriol plus paclitaxel resulted in a significant decrease in surviving fraction compared to paclitaxel or calcitriol alone. Thus, *in vivo* antitumor activity is increased by pre-treatment with calcitriol followed by paclitaxel.

**In vivo effects of calcitriol and paclitaxel as measured in tumor growth inhibition assays.**

To determine whether an increase in clonogenic cell kill *in vitro* was associated with actual differences in tumor growth, SCC (Fig. 3A) or PC-3 (Fig. 3B) tumor-bearing mice were treated with either saline, calcitriol or paclitaxel alone, or calcitriol in combination with paclitaxel. We utilized a schedule of daily × 3 doses of calcitriol with paclitaxel administered on day 3. As shown in Fig. 3, paclitaxel had no significant activity when used as a single agent therapy in either model. In SCC, where calcitriol alone exhibited cytostatic activity, combination with paclitaxel resulted in significant tumor regression (Fig. 3A). A significant increase in anti-tumor activity was also observed when PC-3 tumor-bearing mice were pre-treated with calcitriol followed by paclitaxel (Fig. 3B). In this model, significant inhibition of tumor growth was maintained for greater than two weeks.

After day 24, PC-3 tumors in animals treated with calcitriol plus paclitaxel exhibited a growth rate comparable to controls. Re-treatment of these animals with calcitriol plus paclitaxel
on days 29-31 resulted in a decrease in the rate of tumor growth, as evidenced by a change in the slope of the growth curve for days 32-38 (Fig. 3C). These data indicate that previously treated tumors remain responsive to calcitriol plus paclitaxel and that improved antitumor activity is achieved by repeated cycles of therapy.

Statement of Work - Specific Aim 2: **To determine the role of calcitriol in the induction of apoptosis and in changes in calcium**

Modulation of cell cycle parameters and apoptosis by calcitriol and paclitaxel.

Diverse mechanisms have been proposed to explain the antitumor activities of calcitriol and paclitaxel in prostate cancer cells. Calcitriol antiproliferative activity has been linked to its ability to promote G_0/G_1 arrest via modulation of the cdk inhibitor p21 in LNCaP cells. In contrast, the antiproliferative activity of paclitaxel in PC-3 cells has been attributed to its ability to induce apoptosis via phosphorylation and inactivation of the apoptosis suppressor protein, Bcl-2 (13). To investigate whether calcitriol and paclitaxel regulate the same or different antiproliferative pathways, PC-3 cells were treated *in vitro* with either EtOH vehicle control, calcitriol alone, paclitaxel alone, or the combination of calcitriol plus paclitaxel. At various times, cells were harvested and analyzed for cell cycle alterations and the induction of apoptosis.

At each of the time points examined, treatment with calcitriol alone resulted in little change in the percentage of cells in G_0/G_1 compared to EtOH control (Table 1), which was not

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time</th>
<th>% events with designated DNA Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sub-G_0/G_1</td>
<td>G_0/G_1</td>
</tr>
<tr>
<td>EtOH</td>
<td>24h</td>
<td>2.98</td>
</tr>
<tr>
<td>calcitriol</td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td>Ptx</td>
<td></td>
<td>65.4</td>
</tr>
<tr>
<td>calcitriol + Ptx</td>
<td></td>
<td>58.3</td>
</tr>
</tbody>
</table>
accompanied by an increase in the cyclin dependent kinase inhibitor, p21 (Fig. 4A). In multiple experiments, calcitriol treatment resulted in a decrease in p21 expression after 96h. Treatment with paclitaxel alone induced the accumulation of nuclei or nuclear fragments with a sub-G_0/G_1 DNA content, which are likely to be apoptotic. In cells treated with 100 nM paclitaxel, the cdk inhibitor p21 was induced within 24h and remained elevated until 48h post-treatment (Fig. 4A). Compared to paclitaxel alone, the number of nuclei/nuclear fragments with sub-G_0/G_1 DNA content was slightly, but consistently, reduced by treatment with the combination of calcitriol plus paclitaxel (Table 1). Calcitriol did not modulate the induction of p21 by paclitaxel (Fig. 4A). These data indicate that calcitriol does not modulate the magnitude or kinetics of paclitaxel induced cell cycle changes. Similar results were obtained at either lower (10 nM) or higher (500 nM) doses of paclitaxel (data not shown).

The appearance of a sub-G_0/G_1 peak in cell cycle analysis is a frequently used indicator of apoptosis. To further investigate whether paclitaxel induces apoptosis in PC-3 cells under the experimental conditions employed, and whether this response is modulated by calcitriol, cell lysates were prepared after in vitro treatment with EtOH vehicle control, calcitriol alone, paclitaxel alone, or the combination of calcitriol plus paclitaxel and analyzed by Western blot for processing of pro-caspase-3 (Fig. 4B) and cleavage of the caspase-3 substrate, PARP (Fig. 4c). Calcitriol alone did not modulate pro-caspase-3 or PARP expression in PC-3 cells. However, expression of both proteins decreased with similar kinetics in cells treated with paclitaxel for 48-96h. A comparable time-dependent reduction in pro-caspase-3 and PARP expression was observed in cells treated with the combination of paclitaxel and calcitriol. These data demonstrate that paclitaxel induces apoptosis in PC-3 cells which does not appear to be further accelerated by calcitriol.

Paclitaxel induces apoptosis in PC-3 cells by promoting the phosphorylation and inactivation of the apoptotic suppressor protein, Bcl-2 (13). We investigated the effects of administering paclitaxel in combination with calcitriol on Bcl-2 expression. Consistent with
modification by phosphorylation, Bcl-2 migrated as a doublet in lysates prepared from PC-3 cells treated with paclitaxel for 24h (Fig. 4D). At subsequent times, Bcl-2 was decreased to nearly undetectable levels in cells treated with paclitaxel alone. Calcitriol alone did not regulate Bcl-2 nor did it alter the effects of paclitaxel on Bcl-2 expression.

Modulation of calcitriol-induced hypercalcemia by paclitaxel or the bisphosphonate, Zoledronate.

We have previously described that paclitaxel reduces calcitriol-induced hypercalcemia in rats and mice when paclitaxel is administered on day 3 of a QDX3 schedule of calcitriol. There are preclinical data indicating that bisphosphonates block calcitriol-mediated osteolysis (14). Since bisphosphonates clearly show evidence of skeletal protection and improved survival in women with advanced breast cancer and are being actively evaluated in prostate cancer, we have initiated studies of bisphosphonates and paclitaxel as modulators of the only known toxicity of calcitriol, hypercalcemia: The following table summarizes data from our prior work with paclitaxel and calcitriol and presents our preliminary findings with the bisphosphonate, Zoledronate kindly provided by Novartis Pharmaceuticals:

<table>
<thead>
<tr>
<th>Serum calcium (mean +/- S.D.) at:</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.9 +/-0.2</td>
<td>11.1 +/-0.1</td>
<td>10.9 +/-0.3</td>
</tr>
<tr>
<td>Calcitriol (C)</td>
<td>16.9 +/-0.7</td>
<td>18.8 +/-1.4</td>
<td>15.6 +/-0.4</td>
</tr>
<tr>
<td>Paclitaxel (P)</td>
<td>10.6 +/-0.4</td>
<td>11.1 +/-0.7</td>
<td>10.6 +/-0.6</td>
</tr>
<tr>
<td>Zoledronate (Z)</td>
<td>9.5 +/-0.5</td>
<td>9.3 +/-0.2</td>
<td>10.1 +/-0.2</td>
</tr>
<tr>
<td>C+P*</td>
<td>ND</td>
<td>17.1 +/-12.5</td>
<td>12.9 +/-0.8</td>
</tr>
<tr>
<td>C+Z**</td>
<td>12.8 +/-0.3</td>
<td>13.2 +/-0.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

Calcitriol was administered 0.75μg hour 0, 24 and 48
Paclitaxel was administered 10 mg/kg hour 48
Zoledronate was administered 10 mg/kg, hour 0
These data clearly indicate that both paclitaxel and Zoledronate block calcitriol-induced hypercalcemia. Preliminary studies of Zoledronate + calcitriol in animals bearing SCC tumors show no effect of Zoledronate on calcitriol-antitumor activity and it is important to reiterate that our data clearly indicate that calcitriol potentiates the antitumor activity of paclitaxel.

Statement of Work- Specific Aim 3: To evaluate the toxicities, MTD, pharmacokinetics of paclitaxel and calcitriol in patients with advanced prostate cancer

Clinical trial results to date.

We have treated 13 patients on the clinical trial proposed in this grant. The following is the treatment schedule utilized:

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcitriol</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>80mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>80mg</td>
</tr>
<tr>
<td>PK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Paclitaxel is given at a dose of 80mg/sq m D1, D10, D17 and D23, every 6 weeks Calcitriol is administered weekly by mouth according to the following dose escalation scheme: 4, 6, 8, 11, 15, 20, 26, 34 and at 30% increments for subsequent escalation in cohorts of 3-6 patients.

We have entered 13 patients on trial and have completed escalations through 11 μg p.o. QDX3 weekly + paclitaxel. Mild myelosuppression has been seen which has not been dose limiting; no patient has experienced a serum calcium (corrected) >11mg/dL. We are conducting a concurrent phase 2 trial in androgen-independent prostate cancer of calcitriol (Monday, Tuesday and Wednesday) + dexamethasone (4mg Sunday, Monday, Tuesday and Wednesday). In this study we have treated 24 patients with 12μg QDX3 of calcitriol weekly (total weekly dose 36μg/week) for at least one month. No patient has had hypercalcemia. Our prior studies of calcitriol alone administered on a daily oral basis indicate that at least 30% of individuals will develop...
hypercalcemia at a weekly dose of 7.5-14µg/week. In individuals treated with calcitriol to retard the development of osteoporosis, doses of calcitriol of >14µg/week are frequently associated with hypercalcemia. Hence, failure to observe hypercalcemia in the present paclitaxel + calcitriol trial despite administering a weekly dose of calcitriol substantially higher than one would expect to be able to administer if calcitriol were administered daily may be attributable to the intermittent schedules of calcitriol employed and/or the effects of either dexamethasone or paclitaxel in modulating calcitriol-induced hypercalcemia.

Blood samples to assess plasma pharmacokinetics of paclitaxel when administered on Day 1 with the initiation of calcitriol and on Day 10, after 3 days of calcitriol have been collected, as have samples for assessment of calcitriol pharmacokinetics. These samples have not yet been analyzed; the initial batch analysis is planned for this Fall. No difference in toxicity between week 1 and week 2 of paclitaxel as been noted, suggesting that there is no reduction in paclitaxel clearance associated with calcitriol administration; such a conclusion is obviously premature, however. Conclusions regarding interactions of calcitriol and paclitaxel must await the completion of careful analyses of calcitriol and paclitaxel pharmacokinetics.

**Methods utilized:**

**Tumor cells and model systems.** The human prostatic adenocarcinoma cell line PC-3 was obtained from the American Type Culture Collection (Manassas, VA). PC-3 cells are VDR⁺, androgen-independent and p53 null. For *in vitro* studies, cells were grown in F-12K medium supplemented with 10% FCS and 2 mM L-glutamine. *In vivo*, tumors were routinely produced by subcutaneous inoculation of $2 \times 10^6$ log-phase tissue culture cells mixed 1:1 with Matrigel in the
right flank of nude mice (Taconic Farms, Germantown, NY). Studies were initiated 8 days later when tumors were palpable.

SCCVII/SF is a moderately well differentiated squamous cell carcinoma derived from a spontaneously arising tumor of the C3H mouse (15). Murine SCCVII/SF squamous cell carcinoma cells were obtained from Dr. Karen Fu (University of San Francisco, CA). They were transplanted as previously described in 6- to 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 medium plus 15% FCS (HyClone Laboratories, Inc., Logan, UT) and passed only twice before being returned to the animals. In vivo, tumors were routinely produced by subcutaneous inoculation of $5 \times 10^5$ log-phase tissue culture cells in the right flank of each mouse. Studies were initiated approximately 9 days later when the tumors were palpable.

**Chemicals and reagents.** Calcitriol (Hoffmann-LaRoche, Nutley, NJ) was reconstituted in 100% ethyl alcohol and stored protected from light under a layer of nitrogen gas at -70 C. All handling of calcitriol was performed with indirect lighting. Paclitaxel (Bristol-Myers Squibb, Princeton, New Jersey) 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) and monoclonal mouse anti-human Bcl-2 (DAKO Carpinteria, CA). Monoclonal anti-caspase-3 and polyclonal rabbit anti-p21 were from PharMingen (San Diego, CA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Life Sciences (Arlington Heights, IL) and Promega (Madison, WI), respectively.

**Clonogenic tumor cell survival assays.** Tumor cells were incubated for 24 hours in T25 flasks (Corning Costar Corp., Cambridge, MA) with or without calcitriol. Cells were then either left untreated, or were treated with various concentrations of paclitaxel for an additional 24 hours. The cells were then harvested, counted, and re-plated at various dilutions into 6-well tissue culture plates (Corning Costar). After a 7-day incubation at 37 C in a humidified atmosphere containing 5% CO$_2$, cell monolayers were washed with saline, fixed with 100% methanol, and
stained with 10% Giemsa. Colonies were counted with the use of a light microscope. To calculate the surviving fraction, the cloning efficiency of treated cells was divided by the cloning efficiency of untreated, control cells.

**In vivo clonogenic assay.** The *in vivo* effect of paclitaxel with and without calcitriol on clonogenic tumor cells was determined by a modification of the *in vivo* clonogenic cell survival assay as described previously (16). Briefly, mice with 9-day squamous cell carcinomas (three to five animals per group) were treated with saline or 2.5 μg calcitriol each day for 3 days. On day 3, mice also received varying doses of paclitaxel. Twenty-four hours after the last injection, the animals were sacrificed and their tumors excised. Aliquots of minced tumor were enzymatically dissociated for 60 minutes at room temperature with a mixture of type I collagenase, deoxyribonuclease, and EDTA. Viable tumor cells were then plated at various dilutions in 6-well tissue culture plates. After incubation for 7 days, colonies were counted and the numbers of clonogenic cells per gram of tumor was calculated. 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 PC-3 or SCC tumors. Animals were treated for 3 days with single, daily injections of saline or calcitriol. On day 3, animals also received a single intraperitoneal injection of paclitaxel. Tumor measurements were obtained using calipers prior to initiating treatment (initial tumor volume) and every day thereafter. Tumor volumes were calculated by the following formula: volume = (length × width²)/2.

**Preparation of cell lysates and Western blot analysis.** Protein was extracted from *in vitro* treated cells using lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 0.6 mM PMSF, and 5 g/ml leupeptin). Cell monolayers were washed twice with PBS, and 200 ml of lysis buffer was added per T25 flask. Flasks were rocked for 30 min at 4 C. Lysates were transferred to 1.5 ml Eppendorf tubes and clarified by centrifugation at 13,000 rpm for 10 min at 4
C. 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 -80°C before use. Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions, and then electrophoretically transferred to poly(vinylidene difluoride) (PVDF) membranes (NEN Life Science Products, Boston, MA) overnight at 4°C. At room temperature, membranes were blocked for a minimum of 1h in a 5% w/v solution of non-fat milk in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween-20) then incubated for 1h with primary antibody. The blots were washed 3x in TBST and subsequently incubated with secondary antibody conjugated with horseradish peroxidase for 1h. The blots were again washed and the proteins detected using Renaissance Western blot chemoluminescence reagents (NEN Life Science Products).

**Cell cycle analysis.** The effect of various treatments on tumor cell cycle was determined using a detergent-trypsin method to stain nuclei for DNA analysis using flow cytometry as described by Vindelov et al (17). Tumor cells were seeded in T25 flasks at varying concentrations determined to produce 80-90% confluence upon harvest after the desired incubation interval. Twenty-four hours after plating, the cells were either left untreated or were treated with calcitriol alone, paclitaxel alone, or the combination of calcitriol plus paclitaxel for varying lengths of time at 37°C. Following treatment, the media containing floating cells was harvested, and trypsin was added to the adherent cell monolayer. The resulting single cell suspension was diluted with fresh media, pooled with the floating cells, and centrifuged for 5 min at 800 rpm. The cell pellet was resuspended in citrate buffer (250 mM sucrose, 40 mM citrate, 5% DMSO) and flash frozen in dry ice/EtOH before storage at -80°C. Nuclei were subsequently isolated and stained with propidium iodide as described. The stained nuclei were filtered using 30 m nylon mesh and analyzed using a FACSTAR flow cytometer.

**Clinical Trial:** In conjunction with these preclinical studies we are conducting a phase one clinical trial of paclitaxel, 80mg/sqm, administered weekly for four weeks, followed by two weeks of rest. Calcitriol is administered orally daily for three days each week starting two days prior to the weekly paclitaxel dose, in escalating doses in cohorts of three patients per dose.
escalation level. The starting dose for this phase one trial was 4µg QD X3, weekly. Serum calcium, phosphorus, creatinine and CBC are monitored weekly, physical exam is performed q6 weeks and antitumor effect is assessed monthly. This trial is open to patients with advanced solid tumors for whom no curative or more effective therapy is available.
Key Research Accomplishments

- Pre-treatment with calcitriol enhances paclitaxel – mediated anti-tumor activity in vitro and these effects are schedule dependent.

- Calcitriol enhances paclitaxel – mediated anti-tumor effects in vivo by both the excision clonogenic assay as well as by actual reduction in tumor volume in animals with established tumors

- Repeated treatment with cycles of calcitriol (daily x3) and paclitaxel on day 3 prolonged tumor growth inhibition.

- Calcitriol did not appear to enhance paclitaxel – mediated apoptotic and cell cycle effects

- Paclitaxel and biphosphohates decrease calcitriol – mediated hypercalcemia

- Patients with advanced cancer are treated with weekly cycles of calcitriol (daily x 3) plus paclitaxel (on day 3) and evaluated for toxicity, determination of MTD and pharmacokinetic effects. To date, the MTD has not been reached and the trial continues to accrue.
Reportable Outcomes

Manuscripts:


Abstracts:


Presentations:

Invited speaker: Donald L. Trump, M.D. vitamin D Workshop, San Francisco, CA November 30, 1998 “Clinical application of vitamin D (calcitriol) and its analogs for cancer”.

-19-
Presentations (Cont’d):

Invited speaker: Donald L. Trump, M.D. CaPCURE, Lake Tahoe, NV, September 1998 “Clinical and Pre-Clinical Evaluation of 1,25 dihydroxy vitamin D$_3$ in combination with paclitaxel in prostate cancer”.

Invited speaker: Donald L. Trump, M.D. CaPCURE, Lake Tahoe, NV, October 1999 “vitamin d and vitamin D Analogues: pre-clinical and clinical studies in prostate cancer.”
Conclusions

We and others have demonstrated the antiproliferative activity of calcitriol in prostatic adenocarcinoma cell lines as well as other tumor systems in vitro and in vivo (6-8). Antiproliferative effects of calcitriol have been 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 (18). Despite its promising preclinical information and indications of activity in these patients, no responses were observed in 6 patients with advanced prostate cancer treated in our phase I trial of subcutaneous calcitriol at doses of 2-8 μg QOD (19), nor in our phase II trial of oral calcitriol at doses of 0.5-1.5 μg QD (20). In an effort to develop better therapies for advanced malignancy, we investigated the effect of combining calcitriol with the microtubule stabilizing agent paclitaxel, which on its own displayed little activity against HRPC. Our data clearly indicate that there is an increase in antitumor activity in prostatic adenocarcinoma using calcitriol in combination with paclitaxel. This enhancement occurs both in vitro and in vivo as measured in clonogenic assays and tumor outgrowth studies. In vitro analyses revealed that calcitriol does not modulate the apoptosis-inducing activity of paclitaxel, suggesting that these two agents function in separate, but complementary pathways to control tumor proliferation.

We have also observed that calcitriol enhances paclitaxel antiproliferative activity in vitro and in vivo in the murine squamous cell model, SCCVII/SF (see appendix). A previous report indicates that these cells are relatively resistant to paclitaxel in vivo at a concentration of 40 mg/kg (21). We found that although paclitaxel (20 mg/kg) has little activity when administered to tumor-bearing mice as a single agent, it becomes a potent antitumor agent following pre-treatment with calcitriol (Figs. 1B, 3B &C). These data suggest that calcitriol and paclitaxel combination therapy may be useful even in the treatment of tumors thought to be relatively paclitaxel insensitive.
The clinical use of calcitriol is restricted by its dose-limiting toxicity, hypercalcemia. However, a variety of calcitriol analogs, including Ro 23-7553 and EB1089, have been described which possess antiproliferative activity without as strong a tendency to induce hypercalcemia (22,23). It has recently been shown that EB1089, when combined with paclitaxel, inhibits the growth of MCF-7 breast cancer cells in vivo (24). It is possible that significant antitumor activity without hypercalcemic side effects can also be obtained in prostate cancer models and in patients with HRPC using calcitriol analogs in combination with paclitaxel.

Paclitaxel stabilizes microtubules and prevents their assembly into structures necessary for mitosis, resulting in accumulation of cells in the G2/M phase of the cell cycle and apoptosis. Calcitriol has been shown to induce G0/G1 arrest in prostate cancer cells and to inhibit their invasiveness in vitro (11,25). Therefore, it was anticipated that the increase in antiproliferative activity observed for the combination of calcitriol and paclitaxel would likely result from effects on separate anti-proliferative pathways. To test this hypothesis, we examined whether calcitriol modulated the effects of paclitaxel in PC-3 cells.

Within 24h, paclitaxel induced expression of p21 and mediated down-modulation of Bcl-2 (Figs. 4A & D). Both of these changes also occur in MCF-7 cells treated with paclitaxel and may result from activation of raf-1 kinase (26). Loss or inactivation of Bcl-2 in prostate cancer cells following paclitaxel administration has been proposed to promote cell death by shifting the intracellular balance of death regulators in favor of pro-apoptotic molecules such as Bax (13). Consistent with these findings, we observe a significant level of apoptosis, as measured by the loss of full-length procaspase-3 and PARP, within 72h of treatment of PC-3 cells with paclitaxel (Fig. 4B & C). Calcitriol had no effect on paclitaxel modulation of the molecular parameters measured in this study, supporting the hypothesis that these two agents function in separate, but complementary pathways to control tumor proliferation.

One might argue that we did not observe an effect of calcitriol on paclitaxel activity in our molecular studies because of the concentration of paclitaxel used. However, we consider this an unlikely possibility since similar results were obtained when the paclitaxel concentration was
decreased to 10 nM or increased to 500 nM (data not shown). Furthermore, these paclitaxel concentrations were in the range used in our in vitro clonogenic assays, where calcitriol clearly increased paclitaxel activity.

To date our studies indicate that calcitriol clearly potentiates the cytotoxic activity (in vitro and in vivo, in PC-3 and SCC). It was hoped that studies of apoptotic markers, cell cycle effects and p21/p27 modulation would shed light on the mechanisms underlying the potentiation of paclitaxel by calcitriol. Mechanistic studies continue exploring our recent observations that calcitriol inhibits MAPK, evaluating the effect of calcitriol on intracellular calcium fluxes as well as the effect of calcitriol on expression of retinoid receptors: RXR is the heterodimeric partner of VDR; heterodimerization is required for calcitriol-VDR mediated effects. Our clinical trial clearly indicates that substantial doses of calcitriol may be administered with paclitaxel without difficulty; this trial will continue to determine the MTD of calcitriol in this setting. We are confident that the continuation of these studies will shed light on mechanisms of interaction of these two agents and provide information regarding their use in combination in patients with prostate cancer.

An alternative possibility is that calcitriol enhances paclitaxel activity by modulating cellular factors we did not measure directly, which themselves may reduce the threshold for induction of apoptosis by paclitaxel. For example, calcitriol and the calcitriol analog EB1089 increase the expression of insulin-like growth factor binding protein 5 (IGFBP) in the MCF-7 breast cancer cell line (27) and IGFBPs-2, 3, 4 and 5 in normal rat prostate (28). IGFBPs interfere with the growth-promoting activities of insulin-like growth factor 1, or may themselves induce apoptosis independent of their binding to insulin-like growth factors (29). If calcitriol upregulates IGFBPs in our experimental system, this could reduce the survival signal provided by IGF-1, rendering these cells more sensitive to apoptosis induced by paclitaxel. The sensitivity of PC-3 cells to the antiproliferative action of IGFBP-3 has been reported (29), supporting this hypothesis.
References:


(10) Hedlund, T.E., K.A. Moffatt, and G.J. Miller: Stable expression of the nuclear vitamin D receptor in the human prostatic carcinoma cell line JCA-1: evidence that the antiproliferative
effects of 1α,25-dihydroxyvitamin D₃ are mediated exclusively through the genomic signaling pathway. Endocrinology 137:1554-1561, 1996.


Appendices:  Figures for Progress Report
           Submitted Manuscript
           Abstracts
Figure legends:

Fig. 1. Dose-response curve of (A) SCC and (B) PC-3 cells treated in vitro with calcitriol alone, with varying doses of paclitaxel alone, pre-treated 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. In pre-treated cells, calcitriol was added 24h prior to paclitaxel. Cells were exposed to paclitaxel for 24h. SCC cells and PC-3 cells were treated with 4.0 nM calcitriol and 5 μM calcitriol, respectively. Each point represents the mean surviving fraction as determined by counting triplicate wells; error bars represent the 95% confidence intervals.

Fig. 2. Paclitaxel mediates dose-dependent clonogenic cell kill when administered alone or in combination with calcitriol. SCC tumor bearing mice were treated with saline or 2.5 μg calcitriol each day for 3 days. On the third day, mice also received varying intraperitoneal doses of paclitaxel. 24h later, tumors were harvested, dissociated, and plated in the excision clonogenic assay. Colonies were enumerated 7 days after plating. Each point represents the mean surviving fraction for total clonogenic cells per gram of tumor (3 to 5 mice per treatment group).

Fig. 3. Calcitriol plus paclitaxel combination therapy has increased anti-tumor activity in the SCC and PC-3 animal tumor models. (A) C3H mice bearing palpable, subcutaneous SCC tumors were treated with either saline, 1.25 μg calcitriol daily for 3 days, 20 mg/kg paclitaxel on day 3, or the combination of 1.25 μg calcitriol daily for 3 days plus 20 mg/kg paclitaxel on day 3. Both agents were administered i.p. (B) Nude mice bearing palpable, subcutaneous PC-3 tumors were treated with either saline, 0.75 μg calcitriol daily for 3 days, 10 mg/kg paclitaxel on day 3, or the combination of 0.75 μg calcitriol daily for 3 days plus 10 mg/kg paclitaxel on day 3. Both agents were administered i.p. (C) Animals previously treated with 1,25-D3 plus paclitaxel on days 8-10 were treated with a second cycle of therapy on days 29-31. Tumor volumes were calculated as described in the Methods and were recorded prior to initiating treatment (initial tumor volume).
and on each of the days indicated. For a given tumor, fractional tumor volume was calculated by dividing its volume on a given day by its initial volume. Data points represent the mean fractional tumor volume for 8 to 10 animals per group.

**Fig. 4.** Paclitaxel modulates expression of the cdk inhibitor p21 and apoptosis associated proteins caspase-3, PARP and Bcl-2 in PC-3 cells. Whole cell lysates were prepared from subconfluent PC-3 cells treated *in vitro* with EtOH, 5 μM calcitriol, 100 nM paclitaxel, or 5 μM calcitriol + 100 nM paclitaxel for 24-96h. Protein from each cell lysate was resolved on polyacrylamide gels under denaturing conditions, transferred to PVDF membrane, and analyzed by Western blot using antibodies raised against (A) p21, (B) Caspase-3, (C) PARP, and (D) Bcl-2. Protein expression levels were quantitated by densitometry and normalized to EtOH control at each time point. Similar results were obtained in a second, independent experiment.
Figure 2

Surviving fraction/gm tumor

Paclitaxel (mg/kg)
Enhancement of paclitaxel antitumor activity in squamous cell carcinoma and prostatic adenocarcinoma by 1,25-dihydroxycholecalciferol (1,25-D$_3$)$^1$


From the Departments of Pharmacology*, Medicine†, and Surgery§ and The University of Pittsburgh Cancer Institute‡
University of Pittsburgh, School of Medicine
Pittsburgh, PA 15213

Key Words: paclitaxel, dihydroxycholecalciferol, squamous cell carcinoma, prostatic adenocarcinoma

$^1$Abbreviations: 1,25-dihydroxycholecalciferol (1,25-D$_3$); squamous cell carcinoma (SCC); poly(ADP-ribose) polymerase (PARP); vitamin D receptor (VDR); ethanol (EtOH); poly(vinylidene difluoride) (PVDF).

$^2$Corresponding Author: Pamela A. Hershberger, Ph.D.
University of Pittsburgh
Dept. of Pharmacology
W1002 Biomedical Science Tower
Pittsburgh, PA 15213
(412) 648-2344
Fax (412) 648-9856
Abstract

Background: The antiproliferative activity of 1,25-dihydroxycholecalciferol (1,25-D₃) in squamous cell carcinoma is associated with decreased p21Waf1/Cip1 (p21) expression (1). Recent studies indicate p21 reduction increases tumor cell sensitivity to paclitaxel (2), (3). We therefore examined whether paclitaxel cytotoxicity is increased by combination with 1,25-D₃.

Methods: The effects of 1,25-D₃ and paclitaxel on growth of the murine squamous cell carcinoma (SCCVII/SF) and the human prostatic adenocarcinoma (PC-3) in vitro and in vivo were determined by clonogenic tumor cell assay and by monitoring tumor growth. The in vitro effects of 1,25-D₃ and paclitaxel on p21, Bcl-2, caspase-3, and poly(ADP-ribose) polymerase (PARP) in PC-3 were evaluated by Western blot. Results: Treatment of SCC or PC-3 in vitro with 1,25-D₃ prior to paclitaxel significantly reduced clonogenic survival compared to either agent alone. Treatment of SCC- or PC-3-tumor bearing mice with 1,25-D₃ prior to paclitaxel resulted in substantially greater growth inhibition than was achieved with either agent alone. Treatment in vitro with 1,25-D₃ resulted in a time-dependent decrease in p21 in PC-3. Paclitaxel induced apoptosis in PC-3 as evidenced by the time-dependent loss of procaspase-3 and full-length PARP. Paclitaxel effects were associated with induction of p21 and loss of Bcl-2. None of molecular alterations induced by paclitaxel were further modulated by 1,25-D₃. Conclusions: Although 1,25-D₃ and paclitaxel each display activity in SCC and PC-3, antiproliferative effects are greatly increased by combination of these agents. Effects are schedule dependent; optimal activity is observed when cells are pre-treated with 1,25-D₃ followed by paclitaxel. In PC-3, 1,25-D₃ does not modulate the apoptosis-inducing ability of paclitaxel suggesting 1,25-D₃ and paclitaxel function in separate pathways to inhibit tumor growth.
Introduction

In addition to its classical role in bone and mineral metabolism, the seco-steroid hormone, vitamin D₃ (1,25-dihydroxycholecalciferol, (1,25-D₃)) exhibits antiproliferative activity in solid tumor models both in vitro (4), (5), (6), (7), (8), (9), (10) and in vivo (4), (10), (11). 1,25-D₃ binding activates the vitamin D receptor (VDR), a member of the steroid nuclear receptor superfamily, resulting in modulation of the transcription of target genes with promoters containing a vitamin D response element (12). 1,25-D₃ treatment induces expression of the cyclin dependent kinase (cdk) inhibitors, p21Waf1/Cip1 (p21) and/or p27kip1(p27), in breast (13), prostate (14) and pancreatic cancer cell lines (8) in vitro. These proteins block G₀/G₁ progression by binding to and inhibiting cyclin:cdk complexes (15).

We have demonstrated that 1,25-D₃ inhibits the growth of the murine squamous cell carcinoma, SCCVII/SF (SCC) in vitro and in vivo (10). Our in vitro mechanistic studies revealed that 1,25-D₃ induces G₀/G₁ arrest in SCC (16), which is accompanied by an increase in Rb hypophosphorylation, an increase in expression of p27, and a decrease in expression of p21 (1). However, only p21 expression is reduced in SCC tumors harvested from animals treated with therapeutic doses of 1,25-D₃ (1). These findings suggest p21 down-modulation, rather than p27 induction, may be a component of the mechanism by which 1,25-D₃ exerts antiproliferative activity in vivo.

Recent studies indicate a reduction in p21 expression sensitizes tumor cells to apoptosis induced both by DNA damaging agents (17), (18), (19), (20) and microtubule damaging agents such as paclitaxel (3), (21). In MCF-7 breast carcinoma cells, paclitaxel induces p21 expression; treatment of these cells with antisense p21 oligonucleotides increases paclitaxel cytotoxicity (2). Similarly, paclitaxel antitumor activity is increased in vitro and in vivo in HCT116 colon
carcinoma cells deficient in p21 expression compared to cells with wildtype p21 (3). Given the ability of 1,25-D$_3$ 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 1,25-D$_3$ would enhance the antitumor effect of paclitaxel in SCC cells.

We find that antiproliferative activity is optimally increased in vitro by pre-treating SCC cells with 1,25-D$_3$ prior to paclitaxel administration. We further demonstrate that 1,25-D$_3$ significantly enhances paclitaxel antitumor activity in vivo as evaluated in excision clonogenic and tumor growth inhibition assays. Similarly, we observe a significant increase in antiproliferative activity in human prostatic adenocarcinoma, PC-3, using 1,25-D$_3$ in combination with paclitaxel. Analogous to our findings in SCC, a significant time-dependent decrease in p21 expression is observed in PC-3 treated with 1,25-D$_3$. However, the effects of 1,25-D$_3$ on p21 expression are unlikely to account for the increase in paclitaxel antiproliferative activity, suggesting an alternate mechanism for enhancement. Nonetheless, our findings, and the non-overlapping toxicities of 1,25-D$_3$ and paclitaxel, suggest this combination may have utility in the treatment of solid tumors.
Materials and Methods

Tumor cells and model systems. SCCVII/SF is a moderately well differentiated squamous cell carcinoma derived from a spontaneously arising tumor of the C3H mouse (22). SCCVII/SF cells were obtained from K. Fu (University of San Francisco, CA). They were transplanted using the protocol developed by Twentyman et al ( ) in 6- to 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 medium containing penicillin-streptomycin plus 15% FCS (HyClone Laboratories, Inc., Logan, UT) and passed only twice before being returned to animals. In vivo, squamous cell carcinomas were routinely produced by subcutaneous inoculation of $5 \times 10^5$ log-phase tissue culture cells in the right flank of each mouse. Studies were initiated approximately 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 plus 10% FCS and 2 mM L-glutamine. In vivo, adenocarcinomas were routinely produced by subcutaneous inoculation of $2 \times 10^6$ log-phase tissue culture cells mixed 1:1 with Matrigel (Beckton Dickinson, Bedford, MA ) in the right flank of nude mice (Taconic Farms, Germantown, NY). Studies were initiated when tumors were palpable.

Chemicals and reagents. 1,25-D$_3$ (Hoffmann-LaRoche, Nutley, NJ) was reconstituted in 100% ethyl alcohol (EtOH) and stored protected from light under a layer of nitrogen gas at -70 C. All handling of 1,25-D$_3$ was performed with indirect lighting. Paclitaxel (Bristol-Myers Squibb, Princeton, New Jersey) 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) and monoclonal mouse anti-human Bcl-2 (DAKO, Carpinteria, CA). Monoclonal anti-caspase-3 and polyclonal rabbit anti-p21 were from PharMingen (San Diego, CA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Life Sciences (Arlington Heights, IL) and Promega (Madison, WI), respectively.

**In vitro clonogenic tumor cell survival assays.** Tumor cells were incubated for 24 hours in T25 flasks (Corning Costar Corp., Cambridge, MA) with or without 1,25-D_3_. Cells were then either left untreated, or were treated with various concentrations of paclitaxel for an additional 24 hours. The cells were then harvested, counted, and re-plated at various dilutions into 6-well tissue culture plates (Corning Costar). After a 7-day incubation at 37°C in a humidified atmosphere containing 5% CO_2_, cell monolayers were washed with saline, fixed with 100% methanol, and stained with 10% Giemsa. Colonies were counted with the use of a light microscope. To calculate the surviving fraction, the cloning efficiency of treated cells was divided by the cloning efficiency of untreated, control cells.

**In vivo excision clonogenic assay.** The *in vivo* effect of paclitaxel with and without 1,25-D_3_ on clonogenic tumor cells was determined by a modification of the *in vivo* clonogenic cell survival assay. Mice with 9-day squamous cell carcinomas (three to five animals per group) were treated with saline or 2.5 μg 1,25-D_3_ each day for 3 days. On day 3, mice also received varying doses of paclitaxel. Twenty-four hours after the last injection, the animals were sacrificed and their
tumors excised. Aliquots of minced tumor were enzymatically dissociated for 60 minutes at room temperature with a mixture of type I collagenase, deoxyribonuclease, and EDTA. Viable tumor cells were then plated at various dilutions in 6-well tissue culture plates. After incubation for 7 days, colonies were counted and the numbers of clonogenic cells per gram of tumor was calculated. 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 1,25-D$_3$, paclitaxel, or the combination of 1,25-D$_3$ 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 1,25-D$_3$. 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 × width$^2$)/2.

**Preparation of cell lysates and Western blot analysis.** Protein was extracted from *in vitro* treated cells using lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 0.6 mM PMSF, and 5 g/ml leupeptin). Cell monolayers were washed twice with PBS, and 200 μl of lysis buffer was added per T25 flask. Flasks were rocked for 30 min at 4 C. Lysates were transferred to 1.5 ml eppendorf tubes and clarified by centrifugation at 13,000 rpm for 10 min at 4 C. 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 -80 C until use.

Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions, and then electrophoretically transferred to poly(vinylidene difluoride) (PVDF) membranes (NEN Life Science Products, Boston, MA) overnight at 4°C. At room temperature, membranes were blocked for a minimum of 1h in a 5% w/v solution of non-fat milk in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween-20) then incubated for 1h with primary antibody. The blots were washed 3x in TBST and subsequently incubated with secondary antibody conjugated with horseradish peroxidase for 1h. The blots were again washed and the proteins detected using Renaissance Western blot chemiluminescence reagents (NEN Life Science Products).
Results

1,25-D₃ mediated increase in paclitaxel activity in vitro.

To examine the antitumor activity of 1,25-D₃ 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 1,25-D₃ alone, (b) were pre-treated with 1,25-D₃ then treated with paclitaxel, or (c) were treated simultaneously with both agents. As we previously reported, 1,25-D₃ has antitumor activity in SCC, and inhibits clonogenic survival with an IC50 of 4 nM (Fig. 1A and (10)). Paclitaxel alone also inhibits SCC survival with an IC50 of 22.89 nM. However, significantly greater antitumor activity was achieved when 1,25-D₃ was combined with paclitaxel; pre-treatment with 1,25-D₃ reduced the IC50 of paclitaxel to 0.026 nM in these cells, while simultaneous addition of 1,25-D₃ reduced the IC50 of paclitaxel to 2.3 nM. These studies demonstrate antitumor activity in SCC is increased by combining 1,25-D₃ with paclitaxel, and that the optimal schedule for administration is pre-treatment with 1,25-D₃ followed by paclitaxel.

Similarly, we examined whether these agents were effective in inhibiting the growth of human prostatic adenocarcinoma PC-3 cells. These cells were selected for study because they express VDR and are responsive to both 1,25-D₃ (23) and (24), (25) administration. PC-3 cells were treated for 24h with or without 1,25-D₃ and then received no further treatment or were treated for an additional 24h with varying concentrations of paclitaxel. Following treatment, the cells were re-plated at various dilutions and surviving colonies enumerated 7 days later. As shown in Fig. 2, 1,25-D₃ alone had detectable antiproliferative activity in these cells, with an IC50 of 5 μM. Paclitaxel, when used as a single agent, reduced PC-3 clonogenic survival in a
concentration-dependent manner. At each of the paclitaxel concentrations tested, significantly greater growth inhibition was achieved by pre-treating the cells for 24h with 1,25-D_3. 1,25-D_3 was also capable of significantly enhancing paclitaxel-mediated growth inhibition in vitro in the Dunning rat metastatic adenocarcinoma (MLL) model system (data not shown). Therefore, 1,25-D_3 in combination with paclitaxel has increased activity in squamous cell carcinoma and prostatic adenocarcinoma indicating the effects are not cell type specific.

**In vivo effects of 1,25-D_3 and paclitaxel as measured in excision clonogenic and tumor growth inhibition assays.**

To evaluate whether the combination of 1,25-D_3 and paclitaxel has greater in vivo antitumor activity compared to either agent alone, the excision clonogenic assay was used. Tumor-bearing C3H mice were treated for 3 days either with saline or 2.5 μg 1,25-D_3 each day. On the third day, mice were treated with varying intraperitoneal doses of paclitaxel (0 to 60 mg/kg). Twenty-four hours after paclitaxel administration, the tumors were harvested, dissociated, and plated in a clonogenic assay. As shown in Fig. 3A, independent of the concentration of paclitaxel used, the combination of 1,25-D_3 plus paclitaxel resulted in a significant decrease in surviving fraction as compared to paclitaxel or 1,25-D_3 alone. Thus, greater in vivo antitumor activity is achieved in SCC by pre-treatment with 1,25-D_3 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, 1,25-D_3 or paclitaxel alone, or 1,25-D_3 in combination with paclitaxel. We utilized a schedule of daily × 3 doses of 1,25-D_3 with paclitaxel administered on day 3. This regimen was previously shown to maximize
antitumor efficacy while minimizing toxicity or hypercalcemia. As shown in Fig. 3B, paclitaxel had no significant activity when used as a single agent therapy, as previously demonstrated (26). In SCC, where 1,25-D$_3$ alone exhibited cytostatic activity, combination with paclitaxel resulted in significant tumor regression (Fig. 3B).

To determine whether 1,25-D$_3$ plus paclitaxel combination therapy also displays increased antiproliferative activity in vivo in PC-3, tumor-bearing mice were treated with saline, 1,25-D$_3$ or paclitaxel alone, or 1,25-D$_3$ in combination with paclitaxel utilizing the dosing schedule described for SCC. As shown in Fig. 4A, neither paclitaxel nor 1,25-D$_3$ had significant activity when used as a single agent therapy in PC-3. However, significant antitumor activity was observed when PC-3 tumor-bearing mice were pre-treated with 1,25-D$_3$ followed by paclitaxel (Fig. 4A). In this model, significant inhibition of tumor growth was maintained for greater than two weeks.

After day 24, PC-3 tumors in animals treated with 1,25-D$_3$ plus paclitaxel achieved a growth rate comparable to controls as determined by the slope of the growth curve (0.107 vs. 0.102). Re-treatment of these animals with 1,25-D$_3$ plus paclitaxel on days 29-31 resulted in a decrease in the rate of tumor growth, as evidenced by a change in the slope of the growth curve for days 32-38 (m= 0.052) (Fig. 4B). These data indicate that previously treated tumors remain responsive to 1,25-D$_3$ plus paclitaxel and that improved antitumor activity is achieved by repeated cycles of therapy.

**Modulation of cell cycle parameters and apoptosis by 1,25-D$_3$ and paclitaxel.**

Given the findings that loss of p21 sensitizes MCF-7 cells to paclitaxel (2), and 1,25-D$_3$ decreases p21 expression in vitro and in vivo (1), we hypothesized that 1,25-D$_3$ enhances paclitaxel antitumor activity via its effects on p21. To test this hypothesis, PC-3 cells were
treated in vitro with EtOH vehicle control, 1,25-D₃ alone, paclitaxel alone, or the combination of 1,25-D₃ plus paclitaxel. At various times, whole cell lysates were prepared and analyzed for p21 expression by Western blot. It should be noted that we chose to focus on PC-3 for these studies rather than SCC because the molecular effects of paclitaxel in the former cell line are documented (24), (25).

In 4/4 experiments, 1,25-D₃ treatment resulted in a greater than 50% decrease in p21 expression after 96h (Fig. 5 and data not shown). However, in cells treated with 100 nM paclitaxel, the cdk inhibitor p21 was induced within 24h and remained elevated 48h-72h post-treatment (Fig. 5 and data not shown). Paclitaxel induction of p21 has been observed in MCF-7 and PC3M cells and results from c-raf-1 activation (27). 1,25-D₃ did not modulate the induction of p21 by paclitaxel. Since the 1,25-D₃-mediated decrease in p21 occurs at a later time than the paclitaxel induced increase in p21, it is unlikely that 1,25-D₃ enhances paclitaxel cytotoxicity via its effects on p21. Similar results were obtained at either lower (10 nM) or higher (500 nM) doses of paclitaxel (data not shown).

Paclitaxel induces apoptosis in PC-3 cells by promoting phosphorylation and inactivation of the apoptotic suppressor protein, Bcl-2 (24). 1,25-D₃ has also been reported to decrease Bcl-2 in MCF-7 cells (28); raising the possibility that 1,25-D₃ increases the antitumor effect of paclitaxel by further reducing Bcl-2 expression. We therefore investigated whether 1,25-D₃ modulates Bcl-2 expression in PC-3 cells in the presence or absence of paclitaxel. Consistent with modification by phosphorylation, Bcl-2 migrated as a doublet in lysates prepared from PC-3 cells treated with paclitaxel for 24h (Fig. 6). At subsequent times, Bcl-2 was decreased to nearly undetectable levels in cells treated with paclitaxel alone. 1,25-D₃ alone did not regulate Bcl-2
nor did it alter the effects of paclitaxel on Bcl-2 expression. Thus, 1,25-D$_3$ does not increase the antiproliferative activity of paclitaxel in PC-3 cells via its effects on Bcl-2.

Although 1,25-D$_3$ did not affect Bcl-2, it could modulate the expression of other regulators of apoptosis and thereby sensitize cells to paclitaxel. To test this hypothesis, we investigated whether 1,25-D$_3$ treatment accelerates the kinetics of paclitaxel-induced apoptosis. Cell lysates were prepared from PC-3 cells after in vitro treatment with EtOH vehicle control, 1,25-D$_3$ alone, paclitaxel alone, or the combination of 1,25-D$_3$ plus paclitaxel and analyzed by Western blot for processing of procaspase-3 and cleavage of the caspase-3 substrate, PARP (Fig.6). 1,25-D$_3$ alone did not modulate procaspase-3 or PARP expression in PC-3 cells. However, expression of both proteins decreased with similar kinetics in cells treated with paclitaxel for 48-96h. A comparable time-dependent reduction in procaspase-3 and PARP expression was observed in cells treated with the combination of paclitaxel and 1,25-D$_3$. These data demonstrate that paclitaxel induces apoptosis in PC-3 cells which is not further accelerated by 1,25-D$_3$. 

13
Discussion

Clinical implications of the increased antiproliferative activity of 1,25-D$_3$ plus paclitaxel in prostatic adenocarcinoma and squamous cell carcinoma.

Based on epidemiologic findings, Schwartz and Hulka proposed a protective role for 1,25-D$_3$ in prostate cancer (29). Subsequently, we and others demonstrated the antiproliferative activity of 1,25-D$_3$ on prostatic adenocarcinoma cell lines in vitro (4), (5), (14) and in vivo (4), (30). Antiproliferative effects of 1,25-D$_3$ were also observed in a pilot clinical trial in which, in a small set of patients with early, recurrent prostate cancer, 1,25-D$_3$ decreased the rate of PSA rise, resulting in an increase in PSA doubling times (31). Despite its promising preclinical information and indications of activity in these patients, no responses were observed in 6 patients with advanced prostate cancer treated in our phase I trial of subcutaneous 1,25-D$_3$ at doses of 2-8 µg QOD (32), nor in our phase II trial of oral calcitriol at doses of 0.5-1.5 µg QD (33). In an effort to develop better therapies for advanced malignancy, we have investigated the effect of combining 1,25-D$_3$ with the microtubule stabilizing agent, paclitaxel. We show in this report that there is an increase in antitumor activity in prostatic adenocarcinoma using 1,25-D$_3$ in combination with paclitaxel. This enhancement occurs both in vitro and in vivo as measured in clonogenic assays and tumor growth inhibition studies. Based upon these findings, we propose that 1,25-D$_3$ plus paclitaxel combination therapy may have utility in the treatment of hormone refractory disease.

The clinical use of 1,25-D$_3$ is restricted by its dose-limiting toxicity, hypercalcemia. However, a variety of 1,25-D$_3$ analogs, including 7553 and EB1089, have been described which possess antiproliferative activity in vivo without inducing hypercalcemia (10), (30). It has
recently been shown that EB1089, when combined with paclitaxel, inhibits the growth of MCF-7 breast cancer cells in vivo (34). It is therefore likely that significant antitumor activity without hypercalcemic side effects can also be obtained in prostate cancer models and patients with HRPC using 1,25-D$_3$ analogs in combination with paclitaxel. In addition, we observe that paclitaxel significantly decreases 1,25- D$_3$ medicated hypercalcemia (manuscript in preparation). Agents that disrupt or stabilize microtubules can inhibit calcium transport, which may account for this activity (35). The ability to deliver repeated cycles of paclitaxel plus 1,25-D$_3$ without hypercalcemia forms the basis of an ongoing clinical trial where weekly paclitaxel is combined with a daily x3 schedule of 1,25-D$_3$ in patients with advanced cancer in the phase I setting.

We have further demonstrated that 1,25-D$_3$ enhances paclitaxel antiproliferative activity in vitro and in vivo in the murine squamous cell model, SCCVII/SF. A previous report indicates that these cells are relatively resistant to paclitaxel in vivo at a concentration of 40 mg/kg (26). We found that although paclitaxel (20 mg/kg) has little activity when administered to tumor-bearing mice as a single agent, it becomes a potent antitumor agent following pre-treatment with 1,25-D$_3$ (Fig. 3B). These data suggest that 1,25-D$_3$ and paclitaxel combination therapy may be useful even in the treatment of tumors thought to be relatively paclitaxel insensitive.

**1,25-D$_3$ increases the antiproliferative activity of paclitaxel in prostatic adenocarcinoma without modulation of molecular markers.**

In MCF-7 and PC3M cells, paclitaxel administration results in e-raf-1 activation and increased p21 expression (27). Notably, paclitaxel cytotoxicity is increased in MCF-7 cells when p21 expression is specifically perturbed using antisense oligonucleotides (2), suggesting a protective role for p21. Since 1,25-D$_3$ treatment reduces p21 expression in vitro and in vivo in the SCC model (1), we hypothesized that 1,25-D$_3$ might enhance the antitumor activity of
paclitaxel via its effect on p21. To test this hypothesis, we examined whether 1,25-D₃ modulates p21 expression in PC-3 cells in the absence or presence of paclitaxel. Although 1,25-D₃ administration resulted in a consistent reduction in p21 expression in PC-3 cells, this effect was not observed until 96h of treatment. At earlier times (24-48h), paclitaxel was found to induce p21 expression, and this induction was not blocked in the presence of 1,25-D₃. These findings make it unlikely that 1,25-D₃ increases paclitaxel antitumor activity in PC-3 via its effects on p21.

Paclitaxel-mediated apoptosis in LNCaP and PC-3 prostate cancer cells has been associated with Bcl-2 phosphorylation and inactivation (24) and/or down-modulation of the related apoptotic suppressor, Bcl-XL (25). Consistent with these results, we found that within 24h, paclitaxel treatment resulted in phosphorylation and down-modulation of the apoptotic suppressor protein, Bcl-2. Loss or inactivation of Bcl-2 in prostate cancer cells following paclitaxel administration has been proposed to promote cell death by shifting the intracellular balance of death regulators in favor of pro-apoptotic molecules such as Bax (24). In our studies, paclitaxel-mediated changes in the intracellular levels of Bcl-2 temporally precede the loss of full-length procaspase-3 and PARP, suggesting that they may initiate the apoptotic program. 1,25-D₃ had no effect on paclitaxel modulation of Bcl-2 nor did 1,25-D₃ accelerate the kinetics of paclitaxel-induced apoptosis (Fig. 6). These molecular findings lead us to hypothesize that 1,25-D₃ and paclitaxel function in separate, but complementary pathways to control tumor proliferation.

One might argue we did not observe an effect of 1,25-D₃ on paclitaxel activity in our molecular studies because of the concentration of paclitaxel used. However, we consider this an unlikely possibility since similar results were obtained when the paclitaxel concentration was decreased to 10 nM or increased to 500 nM (data not shown). Furthermore, these paclitaxel
concentrations were in the range used in our in vitro clonogenic assays, where 1,25-D_3 clearly increased paclitaxel activity.

Analogous to the findings we report here, other investigators have observed antiproliferative activity of 1,25-D_3 in PC-3 cells (14). However, 1,25-D_3 did not mediate cell cycle arrest in these studies, nor do we find that it induces apoptosis in PC-3. How then does 1,25-D_3 exert antiproliferative activity in PC-3, and how does this activity enhance the effect of paclitaxel? One possibility is that 1,25-D_3 enhances paclitaxel activity by modulating cellular factors we did not measure, which effectively reduce the concentration of paclitaxel required for cytotoxicity. For example, 1,25-D_3 or EB1089 increase expression of insulin-like growth factor binding protein 5 (IGFBP) in the MCF-7 breast cancer cell line (36), IGFBP-6 in human prostate carcinoma cell lines (37) and IGFBPs-2, 3, 4 and 5 in normal rat prostate (38). IGFBPs interfere with the growth-promoting activities of insulin-like growth factor 1, or may themselves induce apoptosis independent of their binding to insulin-like growth factors (39). If 1,25-D_3 upregulates IGFBPs in our experimental system, this could reduce the survival signal provided by IGF-1, rendering these cells more sensitive to paclitaxel. The sensitivity of PC-3 cells to the antiproliferative action of IGFBP-3 has been reported (39), supporting such a hypothesis. Further experimentation is required to examine this possibility. Although the mechanism for 1,25-D_3 enhancement of paclitaxel antitumor activity remains to be determined, our data nonetheless suggest that novel 1,25-D_3/ paclitaxel based combination therapies may be useful in the treatment of solid tumors.
Acknowledgments:

This work was supported by USPHS grant CA67267 from the National Cancer Institute, NIH; USAMRMC grant DAMD 17-98-1-8549, and grants from CaPCURE and the Mary Hillman Jennings Foundation.
References:


comparison of 1,25-dihydroxyvitamin D (Calcitriol) and EB1089. Cancer Epidemiology, Biomarkers, and Prevention 8:241-248, 1999


Figure legends:

**Fig. 1.** Dose-response curve of SCC cells treated *in vitro* with 1,25-D$_3$ alone (▲), with varying doses of paclitaxel alone (⊙), pre-treated with 1,25-D$_3$ followed by paclitaxel (●), or treated simultaneously with 1,25-D$_3$ plus paclitaxel (△), as measured by growth inhibition in the 7-day *in vitro* clonogenic assay. In pre-treated cells, 1,25-D$_3$ was added 24h prior to paclitaxel. Cells were exposed to paclitaxel for 24h. 1,25-D$_3$ was used at a concentration of 4 nM. Each point represents the mean surviving fraction as determined by counting triplicate wells; error bars represent the 95% confidence intervals.

**Fig. 2.** Dose-response curves of PC-3 cells treated *in vitro* with 1,25-D$_3$ alone (▲), with varying doses of paclitaxel alone (⊙), or pre-treated with 1,25-D$_3$ for 24h followed by paclitaxel (●) as measured by growth inhibition in the 7-day *in vitro* clonogenic assay. Cells were exposed to paclitaxel for 24h. 1,25-D$_3$ was used at a concentration of 5 μM. Each point represents the mean surviving fraction as determined by counting triplicate wells; error bars represent the 95% confidence intervals.

**Fig. 3.** 1,25-D$_3$ increases paclitaxel antitumor activity in SCC in vivo. (A) SCC tumor bearing mice were treated with saline (⊙) or 2.5 μg 1,25-D$_3$ each day for 3 days (●). On the third day, mice also received varying intraperitoneal doses of paclitaxel. 24h later, tumors were harvested, dissociated, and plated in the excision clonogenic assay. Colonies were enumerated 7 days after plating. Each point represents the mean surviving fraction for total clonogenic cells per gram of tumor (3 to 5 mice per treatment group). (B) C3H mice bearing palpable, subcutaneous SCC
tumors were treated with either saline (☐), 1.25 µg 1,25-D₃ daily for 3 days (▲), 20 mg/kg paclitaxel on day 3 (○), or the combination of 1.25 µg 1,25-D₃ daily for 3 days plus 20 mg/kg paclitaxel on day 3 (●). Both agents were administered i.p. Tumor measurements were obtained on the days indicated, and fractional tumor volumes calculated as described in Materials and Methods. Data points represent the mean fractional tumor volume for 5 animals per group.

**Fig. 4.** 1,25-D₃ increases paclitaxel antitumor activity in PC-3 in vivo. (A) Nude mice bearing palpable, subcutaneous PC-3 tumors were treated with either saline (☐), 0.75 µg 1,25-D₃ daily for 3 days (▲), 10 mg/kg paclitaxel on day 3 (○), or the combination of 0.75 µg 1,25-D₃ 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. 2B. Data points represent the mean fractional tumor volume for 5 animals per group. (B) Animals previously treated with 1,25-D₃ plus paclitaxel on days 8-10 were treated with a second cycle of therapy on days 29-31.

**Fig. 5.** 1,25-D₃ and paclitaxel modulate expression of the cdk inhibitor p21 in PC-3 cells.

Whole cell lysates were prepared from subconfluent PC-3 cells treated in vitro with EtOH, 5 µM 1,25-D₃, 100 nM paclitaxel, or 5 µM 1,25-D₃ + 100 nM paclitaxel for 24-96h. Protein from each cell lysate was resolved on polyacrylamide gels under denaturing conditions, transferred to PVDF membrane, and analyzed by Western blot using anti-p21 antibodies. Protein expression levels were quantitated by densitometry and normalized to EtOH control at each time point. A representative experiment is shown.
Fig. 6. Paclitaxel modulates expression of apoptosis associated proteins Bcl-2, caspase-3, and PARP in PC-3 cells. Whole cell lysates were prepared from subconfluent PC-3 cells treated in vitro with EtOH, 5 μM 1,25-D₃, 100 nM paclitaxel, or 5 μM 1,25-D₃ + 100 nM paclitaxel for 24-96h. Protein from each cell lysate was resolved on polyacrylamide gels under denaturing conditions, transferred to PVDF membrane, and analyzed by Western blot using antibodies raised against Bcl-2, caspase-3, or PARP. A representative experiment is shown.
Days post-treatment vs. Paclitaxel (mg/kg)

Fractional Tumor Volume

Surviving fraction/gm tumor

Figure 3
Fractional tumor volume

Days post tumor implantation

Figure 4
Relative expression

<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p21

EtOH
1,25-D₃
Ptx
1,25-D₃ + Ptx
EtOH
1,25-D₃
Ptx
1,25-D₃ + Ptx
EtOH
1,25-D₃
Ptx
1,25-D₃ + Ptx

In prostate cancer model systems-human xenograft (PC-3) and rat metastatic Dunning (MLL), we demonstrated that 1,25D3 alone has significant antitumor activity in vitro and in vivo. 1,25D3 treatment of MLL in vitro causes significant G0/G1 arrest, modulates expression of p27 and p21, the cyclin-dependent kinase inhibitors implicated in cell cycle arrest, and induces apoptosis as detected by PARP cleavage (100%) and phosphatidylserine exposure (5%). We also demonstrated in an HCC model that pretreatment with 1,25D3 significantly enhances cisplatin- or carboplatin-mediated antitumor activity in vitro and in vivo. Studies were performed here using PC-3 and MLL to determine the antitumor effect of 1,25D3 with paclitaxel. In vitro, pretreatment with 1,25D3 significantly enhanced paclitaxel clonogenic tumor cell kill. Treatment of established PC-3 and MLL tumors with 1,25D3 (0.5-1μg/day × 3) prior to paclitaxel (10mg/kg) on the third day, resulted in significant inhibition of tumor growth. At day 30 post implant, tumors from PC-3 animals treated with 1,25D3/paclitaxel on day 8 remained 64% smaller than control, 1,25D3 or paclitaxel alone. Effects were maximal when 1,25D3 was given 48 hr (in vitro) or 3 day (in vivo) prior to paclitaxel. In addition, paclitaxel prevented the development of hypercalcemia associated with 1,25D3 administration in MLL rats. These results demonstrate that 1,25D3 exhibits a sequence and time-dependent enhancement of paclitaxel-mediated antitumor activity in prostate models and that paclitaxel blocks 1,25D3 induced hypercalcemia. Supported by NCI grant CA 67267.
1,25D, Dihydroxyvitamin D₃ (1,25D₃) as an Anti-Cancer Agent for Prostate Cancer: Preclinical Studies.

Cassade S. Johnson, Pamela A. Hershberger, Ruth A. Modakowski, and Donald L. Trump, Pittsburgh, PA (Presented by Dr. Trump).

Introduction and Objectives: We demonstrated that 1,25D₃ has significant antitumor activity, modulates cell cycle control, and enhances the efficacy of cytotoxic agents in vitro and in vivo. 1,25D₃ also inhibits metastasis and arrests cells in G₂/M in the Dunning rat prostate (MLL) model. To investigate the mechanism(s) involved, studies were initiated in MLL and the human xenograft prostate (PC-3) tumor models to examine 1,25D₃-mediated effects on expression of p21 and p27, and on the induction of apoptosis. Studies were also initiated to examine whether these effects could be enhanced when 1,25D₃ was administered with paclitaxel.

Methods: Tumor cells were treated with 1,25D₃ at half the IC50, harvested at various time points and examined for expression of p21 and p27 (Western blot analysis) as well as for cleavage of poly ADP-ribose polymerase (PARP) and phosphatidylserine exposure by annexin binding, early markers of apoptosis. PC-3 and MLL tumor-bearing mice were also pre-treated with 1,25D₃ for 3 days with paclitaxel administered on day 3 and monitored for changes in clonogenic cell kill and tumor regrowth delay.

Results: At 24 hr post 1,25D₃ treatment, both p21 and p27 expression decreased in MLL cells with no change in expression at 4 hr. When 1,25D₃ treated MLL were examined for induction of apoptosis, MLL showed nearly 100% cleavage of PARP at 24 hr. Annexin binding by MLL was significantly increased in 1,25D₃ treated cells (34.5%) versus untreated controls (2%). In both MLL and PC-3 models, 1,25D₃ treatment daily on days 0-5 with paclitaxel on day 3, resulted in a significant enhancement of antitumor activity as compared to paclitaxel or 1,25D₃ alone.

Conclusions: These results suggest that the antiproliferative activities of 1,25D₃ may be mediated through an apoptotic pathway and that 1,25D₃ enhances paclitaxel-mediated antitumor effects in prostate tumor models.

Source of Funding: NIH/NCI CA 67267.
Effects of dexamethasone and methylprednisolone on vitamin D receptor (VDR) and retinoid X receptor (RXR) expression: Implications for therapy. Maisel, C.M., Bernardi, R.J., Hershberger, P.A., Trump, D.L., and Johnson, C.S. University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA 15234.

Dexamethasone (DEX) enhances the antitumor activity of vitamin D in vitro and in vivo in a mouse squamous cell carcinoma (SCC) model system. Dex has also been shown to significantly increase vitamin D receptor (VDR) ligand binding and receptor content with no change observed in VDR mRNA or protein levels, suggesting that DEX induces an alternative mechanism of VDR activation. In addition, these effects were specific for DEX and did not occur in vitro or in vivo when vitamin D was combined with methylprednisolone (MP). The retinoid X receptor (RXR) associates with VDR as a heterodimer; RXR:VDR activates vitamin D mediated transcription through vitamin D response element (VDRE) promoter regions. Based on these observations, we examined the effect of vitamin D, alone or with DEX or MP, upon the expression of RXR α, β, and γ by Western blot analysis of whole cell lysates made at various timepoints after treatment. After 48 hr, treatment of SCC with DEX (0.0 μM), MP (2.5 μM), or vitamin D (10 nM) increased RXR α expression as compared to control cells. When vitamin D was combined with DEX, no change was observed as compared to DEX alone. In contrast, when SCC were treated with vitamin D and MP, a decrease in the expression of RXR α was observed compared to MP or DEX alone. No significant effects were observed in the examination of RXR β in any of the treatment groups, and SCC did not express RXR γ. These results demonstrated a differential modulation of RXR α expression with respect to the effects of DEX and MP. Whether effects on RXR expression correlate with DEX-mediated enhancement of antitumor activity remains to be determined. Supported by NIH grant CA87267.
The ability of paclitaxel to significantly decrease vitamin D mediated hypercalcemia. Rueger, RM, Hershberger, PA, Modzalewski, RA, Johnson, CS, and Trump, DL. Deps. of Pharmacology and Medicine, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213. 1,25 dihydroxycholecalciferol (calcitriol) (QD x3) significantly enhances the antitumor activity of paclitaxel (QD x1, day 3) in human PC-3 and rat Dunning MLL prostate model systems. Tumor bearing animals treated with the combination of calcitriol and paclitaxel were not as hypercalcemic as animals treated with calcitriol alone. Hence, studies were initiated to further examine the effect of paclitaxel on calcitriol-induced hypercalcemia. Normal Copenhagen rats and C3H/HeJ mice were given daily x3 calcitriol (0.76ug/rat or mouse) and paclitaxel (10 or 20mg/kg, rats or mice respectively) on day 3; urine and blood were collected at 24, 48, 72, 96, 120 hours post-treatment. In both rats and mice, serum peak calcium and the time of return to normal serum calcium levels were significantly reduced in calcitriol/paclitaxel treated animals as compared to calcitriol alone. Serum creatinine and BUN were elevated in animals treated with calcitriol alone whereas creatinine/BUN were normal in paclitaxel/calcitriol animals with no effect observed in animals treated with calcitriol/paclitaxel. 24 hours after a single injection of calcitriol or calcitriol/paclitaxel, urinary calcium was elevated in animals treated with calcitriol alone with but not in animals treated with the combination. These data indicate that paclitaxel blocks the major toxicity of calcitriol, while calcitriol enhances paclitaxel antitumor effects. Studies exploring the mechanism and clinical application of these interactions are underway. Supported by NIH grant CA 67267 and CaPCURE.
#3528 Apoptotic effects of paclitaxel and calcitriol in rat Dunning MLL and human PC-3 prostate tumor cells in vitro. Modzelewski RA, Hershberger PA, Johnson CS and Trump DL, Depts of Pharmacology & Medicine, University of Pittsburgh Cancer Institute, Pgh, PA 15213.

In Dunning rat (MLL) and xenograft human (PC-3) prostate tumor models in vivo, treatment with calcitriol before paclitaxel resulted in significantly greater antitumor response as compared to either agent alone. Investigations into the effect on apoptosis were undertaken. In MLL, calcitriol decreased Bcl-2, with no change in Bax, resulting in an increased Bax/Bcl-2 ratio favoring cell death. We observed 100% PARP cleavage, 24% annexin binding in MLL cells exposed to calcitriol for 24h. Combination of 10μM calcitriol with 1μM paclitaxel resulted in 100% PARP cleavage at 12h as compared to 24h for paclitaxel or calcitriol alone. PC-3 cells, in contrast, did not show changes in apoptotic markers with the combination compared to either agent alone; correlating with the lack of apoptotic markers with calcitriol exposure alone. These results demonstrate that calcitriol sensitized MLL cells to the apoptotic effect of paclitaxel whereas no effect was observed in PC-3, a model that in vivo is equally sensitive to this combination, and indicates that complex mechanisms of paclitaxel-calcitriol interaction exist. Supported by NIH grant CA-67267 and a grant from CaPUCRE.
Preclinical and Phase I Studies of the Combination of Calcitriol (1,25(OH)2Vitamin D3) and Paclitaxel: Synergistic Antitumor Activity and Reduced Toxicity. Donald L. Trump, Robert M. Ruager, Pamela A. Hershberger, Ruth A. Modzelewski, Chandra Belani, Merrill J. Egorin, Candace S. Johnson, Departments of Medicine and Pharmacology, The University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania.

Preclinical studies indicate that calcitriol (D3) has substantial antitumor effects in studies of human prostate cancer (PC-3) and rodent prostate and squamous cell carcinomas (Dunning and SCC) as well as in human breast and colon cancer and leukemia models. While investigating mechanisms of D3 action, we began studies of D3 in combination with the microtubule-disrupting agent, paclitaxel. In vivo, D3 (0.5ug QDX3) potentiates the growth inhibitory effects of paclitaxel 2-3 fold in the Dunning G and MLL tumors and human PC-3. Studies on the apoptotic effects of this combination demonstrate that D3 significantly decreases the time to PARP cleavage as compared to either agent alone. Also, the hypercalcemic effects of D3 are reduced by paclitaxel treatment. In normal rats 48 and 72 hours following the end of 3 days of treatment with D3 (0.75ug QD ± paclitaxel 10mg/kg on day 3), serum calcium (mg/dL) was: No therapy: 10.9±0.2, 10.6±0.2; D3 alone: 18.8±1.4, 15.6±0.4 and D3+paclitaxel: 15.9±0.7 and 12.9±0.8. Based on these results indicating that D3 potentiates the efficacy of paclitaxel and that paclitaxel blunts the only known toxic effect of D3, we began a phase I clinical trial of D3 (orally, days1,2,3)+paclitaxel (80mg/m2q, day3) weekly X4, q6weeks. D3 is administered in escalating doses in cohorts of 3 patients; the starting dose is 4ug QD X3. On week 1 paclitaxel is given on day 1 while on week 2 and thereafter paclitaxel is given on day 3-this permits exploration of the effect of D3 pretreatment on paclitaxel pharmacokinetics and toxicity. Three patients have been entered without toxicity and escalation continues. These studies suggest the potential for new dosage schedules of D3 and set the stage for the integration of other approaches which may further reduce D3-induced hypercalcemia. Supported by CA67267 and CaP-CURE.

The development of calcitriol and calcitriol analogues as anticancer and immunomodulatory agents would be greatly facilitated by the delineation of surrogate markers of its biologic effects. We are evaluating three enzymatic activities in peripheral blood monocytes which are modulated by calcitriol therapy to serve as accurate and reproducible measures of calcitriol biologic effects: calcitriol oxidation enzymes (OXase), cytidine deaminase (CCDase) and fructose 1,6-bisphosphatase (FBPase). OXase, CCDase and FBPase were assessed in peripheral blood monocytes obtained from six cancer patients at 0, 6, 24 and 48 hr after treatment with either 4 or 6 μg of subcutaneous calcitriol. Consistent changes were seen in CCDase and FBPase activity. CCDase activity at 0, 6, 24 and 48 hrs post calcitriol therapy was 6.6 ± 1.3, 3.2 ± 0.7, 1.9 ± 0.5 and 0.4 ± 0.3 nmoles/min/mg of cellular protein (± SEM), respectively, while the FBPase increased from the pretreatment activity of 0.5 ± 0.2 to 1.5 ± 0.3, 2.2 ± 0.5 and 4.0 ± 1.2 nmoles/min/mg of cellular protein at 6, 24 and 48 hrs, respectively. Changes in 0 vs 48 hr CCDase and FBPase activities were significant with p-value of <0.01 and 0.05, respectively. Although no consistent changes in the capacity of these patients monocytes to oxidize calcitriol was seen, it is noteworthy that the baseline capacity to metabolize calcitriol was 3X greater than that of monocytes of normal volunteers. Changes in these monocytic enzymes were associated with the down regulation of VDR expression. Our results suggest that the inverse ratio of monocytic CCDase to FBPase activity has the potential to be a non-hypercalcemic surrogate marker of biologic effects of calcitriol in cancer patients.