Effects of 12-\(O\)-Tetradecanoylphorbol-13-acetate (TPA) in Combination with Paclitaxel (Taxol) on Prostate Cancer LNCaP Cells Cultured \textit{in vitro} or Grown as Xenograft Tumors in Immunodeficient Mice

Xi Zheng, 1 Richard L. Chang, 1 Xiao-Xing Cui, 1 Gina E. Avila, 1 Vidya Hebbar, 2 Mark Garzotto, 4 Weichung Joe Shih, 5, 6 Yong Lin, 6 Shou-En Lu, 6 Arnold B. Rabson, 3, 6 Ah Ng Tony Kong, 2 and Allan H. Conney 1, 6

Abstract

Purpose: To investigate the effects of 12-\(O\)-tetradecanoylphorbol-13-acetate (TPA) in combination with paclitaxel (Taxol) on prostate cancer cells cultured \textit{in vitro} or grown as tumors in immunodeficient mice.

Experimental Design: Human prostate cancer LNCaP cells in culture were treated with TPA alone or in combination with paclitaxel. NCr immunodeficient mice with well-established LNCaP tumors received i.p. injections with vehicle or with TPA, paclitaxel, or TPA in combination with paclitaxel. The animals either received daily treatment for 5 consecutive days followed by a 2-day intermission, which was repeated for a total of 28 days (experiment 1), or continuous daily treatment for 28 days (experiment 2).

Results: Treatment of LNCaP cells with a combination of TPA and paclitaxel synergistically inhibited the growth and induced apoptosis in cultured LNCaP cells, and this treatment also induced a marked increase in phosphorylated c-Jun-NH\(_2\)-kinase (JNK). In animal experiments, tumor growth occurred in all mice treated with vehicle. When treated with TPA alone, the percentage of animals with some tumor regression was 33% in experiment 1 and 100% in experiment 2. Treatment of animals with paclitaxel alone caused some tumor regression in 17% and 57% of the animals in experiments 1 and 2, respectively. All animals treated with TPA + paclitaxel in both experiments had some tumor regression.

Conclusions: TPA and paclitaxel in combination had a stronger inhibitory effect on the growth of LNCaP cells in culture or as xenograft tumors in immunodeficient mice than either agent alone. Clinical trials with TPA alone or in combination with paclitaxel in patients with prostate cancer may be warranted.

In a preliminary study, our laboratory together with colleagues in China showed beneficial effects from i.v. administered 12-\(O\)-tetradecanoylphorbol-13-acetate (TPA) for the treatment of seriously ill myeloid leukemia patients refractory to other therapy. Decreases in the number of myeloblasts in the bone marrow and in the peripheral blood were observed as well as temporary remission of disease symptoms without serious toxicity (1). In an additional study, i.v. infusions of TPA increased the number of circulating WBC and neutrophils in patients with depressed bone marrow caused by prior treatment with cytotoxic chemotherapeutic drugs (2). The results obtained in both studies and data from a phase I trial with TPA at the Cancer Institute of New Jersey in New Brunswick (3, 4) indicated an acceptable toxicity profile. In cell culture studies, a low clinically achievable concentration of TPA (0.16 nmol/L) in combination with clinically achievable concentrations of all-\textit{trans} retinoic acid (0.1-1 \(\mu\)mol/L), 1a,25-dihydroxyvitamin D\(_3\) (1 nmol/L), or sodium butyrate (100 \(\mu\)mol/L) synergistically inhibited the growth and stimulated the...
differentiation of cultured HL-60 myeloid leukemia cells, suggesting that combinations of these drugs may be more effective than TPA alone for the treatment of refractory myeloid leukemia patients (5).

TPA was shown to inhibit the growth and stimulate apoptosis in human prostate cancer cells (6–8). Treatment of prostate LNCaP cells with clinically achievable concentrations of TPA (1–1.6 nmol/L) resulted in growth inhibition (7, 9 – 11), and treatment of these cells with a severalfold higher concentration of TPA caused apoptosis (7, 9 – 11). In other studies, it was shown that treatment of LNCaP cells with a combination of TPA and γ radiation resulted in a synergistic increase in ceramide synthesis and apoptosis (12). In a recent study, we showed that TPA alone or in combination with all-trans retinoic acid inhibited the growth and induced apoptosis in LNCaP prostate cancer cells cultured in vitro or grown as xenografts in immunodeficient mice, and the combination of TPA and all-trans retinoic acid was more effective than either compound alone (13).

Paclitaxel, a naturally occurring anticancer drug, has gained widespread acceptance as an active broad-spectrum antitumor agent (14). Paclitaxel is one of a few chemotherapeutic agents that is effective in patients with advanced prostate cancer (14, 15). Studies using prostate-specific antigen response as an end point showed the efficacy of paclitaxel in the treatment of metastatic prostate cancer (16 – 18). Treatment of patients with paclitaxel alone or in combination with estramustine resulted in a >50% decrease in serum prostate-specific antigen in 40% to 60% of the treated patients (16 – 18). Unfortunately, patients treated with paclitaxel or other chemotherapeutic drugs experience significant systemic side effects that include neutropenia, mucositis, neurotoxicity, and gastrointestinal effects (15, 17, 18). These adverse effects severely limit the duration and dose of drug treatment that can be administered (14, 18). It is likely that effective combinations of paclitaxel with other anticancer agents will produce synergistic effects so that paclitaxel can be used in lower concentrations and thus decrease toxic side effects.

In the present study, we investigated the effects of TPA and paclitaxel alone or in combination on the growth and apoptosis of cultured LNCaP cells, and we also studied the effects of i.p. injections of TPA alone or in combination with paclitaxel on the growth of well-established LNCaP tumors in NCr immunodeficient mice. We found a synergistic inhibitory effect of TPA and paclitaxel on the growth of cultured LNCaP cells, and we also found an inhibitory effect of TPA and paclitaxel administration on the growth of well-established LNCaP tumors in immunodeficient mice. Administration of a combination of TPA and paclitaxel to these tumor-bearing mice resulted in greater inhibition of tumor growth and more tumor regressions than after administration of TPA or paclitaxel alone.

Materials and Methods

Cell culture and reagents. LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD). TPA was obtained from Alexis Co. (San Diego, CA). Paclitaxel, propylene glycol, polystyrene 80, benzyl alcohol, and ethanol were purchased from Sigma (St. Louis, MO). Matrigel was obtained from BD Biosciences (Bedford, MA). RPMI 1640 tissue culture medium, penicillin-streptomycin, I-glutamine, and fetal bovine serum were from Life Technologues (Grand Island, NY). LNCaP cells were maintained in RPMI 1640 containing 10% fetal bovine serum that was supplemented with penicillin (100 units/mL)-streptomycin (100 μg/mL) and I-glutamine (300 μg/mL) as described (13). Cultured cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and were passed twice a week. In all experiments, LNCaP cells were initially seeded at a density of 1 x 10⁵/mL in a final volume of 2 or 5 mL in 35 mm (for cell growth assay) or 100 mm (for Western blotting) tissue culture dishes, respectively (Corning, NY).

Determination of the number of viable cells. The number of viable cells after each treatment was determined using a hemacytometer under a light microscope (Nikon Optiphot, Nikon, Kanagawa, Japan). Cell viability was determined by the trypan blue exclusion assay, which was done by mixing 80 μL of cell suspension and 20 μL of 0.4% trypan blue solution for 2 minutes. Blue cells were counted as dead cells and the cells that did not absorb dye were counted as live cells. Approximately 400 cells were counted in each sample.

Morphologic assessment of apoptotic cells. Apoptosis was determined by morphologic assessment in cells stained with Hoechst 33342 (19). Briefly, cytospin slides were prepared after each experiment and cells were fixed with acetone/methanol (1:1) for 10 minutes at room temperature, followed by 10 minutes with Hoechst 33342 staining (1 μg/mL in PBS) and analyzed using a fluorescence microscope (Nikon Eclipse TE200, Japan). Apoptotic cells were identified by classic morphologic features, including nuclear condensation, cell shrinkage, and formation of apoptotic bodies (19). At least 200 cells were counted in each sample and the percentage of apoptotic cells was presented.

Flow cytometry. To perform the analysis, 1 x 10⁶ cells were washed with PBS and resuspended in 500 μL stain solution (20 mg/mL polyethylene glycol 8000, 50 μg/mL propidium iodide, 0.1% Triton X-100, and 0.4 mol/L sodium chloride). The mixture was incubated at 4°C overnight in the dark before being analyzed on a Coulter Epics Profile II flow cytometer. Propidium iodide is a fluorescent dye that intercalates into the DNA double helix. Whether cells are in the G₁ phase where they have two copies of their genome (2N) or in the G₂-M phase where they have four copies of their genome (4N), or in S phase where their DNA is between 2N and 4N, can be determined by the amount of propidium iodide that intercalates into the DNA. Pre-G₂-G₁ cells were considered as apoptotic cells. The proportion of cells in each phase of the cell cycle was calculated by using the cytologic software from Coulter Corp. (Miami, FL).

Growth of LNCaP tumors in immunodeficient mice. Male NCr immunodeficient mice (6-7 weeks old) were obtained from Taconic Farms, Inc. (Germantown, NY). The animals were housed in sterile cages and provided with sterilized food and water. Prostate cancer LNCaP cells (2 x 10⁵/mL in 0.1 mL/mouse) suspended in 50% Matrigel (Collaborative Research, Bedford, MA) in RPMI 1640 were injected s.c. into the right flank of the mice. After 4 to 6 weeks, mice with well-established tumors (0.65-1.0 cm long and 0.65-1.0 cm wide) were used in two experiments. In both experiments, animals were injected i.p. with vehicle, TPA alone, paclitaxel alone, or TPA in combination with paclitaxel. Animals in the different experimental groups received the same amount of vehicle (5 μL/g body weight), which consisted of propylene glycol, polysorbate 80, benzyl alcohol, ethanol, and water (40:0.5:1:10:48.5). Tumor size (length x width) and body weight were measured daily. All animal experiments were carried out under an Institutional Animal Care and Use Committee–approved protocol.

Western blotting. After treatment, LNCaP cells were washed with ice-cold PBS and lysed with 800 μL lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 50 mmol/L sodium chloride, 30 mmol/L sodium PPI, 50 mmol/L sodium fluoride, 100 μmol/L sodium orthovandate, 2 mmol/L iodoacetic acid, 5 mmol/L ZnCl₂, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.5% Triton-X100]. The lysates were homogenized by passing through a 23 G needle thrice and kept on ice for 30 minutes. The homogenates were centrifuged at 12,000 x g for 15 minutes at 4°C. The protein concentration of whole cell lysates was determined with a
Bio-Rad protein assay kit. Equal amounts (20 µg) of protein were then resolved on a 10% Criterion Precast Gel (Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane using a semidy transfer system. The membrane was blocked in 5% bovine serum albumin solution for 1 hour at room temperature and subsequently incubated overnight at 4°C with antiphosphorylated c-Jun-NH₂-kine (JNK) primary antibody (1:1,000 dilution, Cell Signaling Technology, Beverly, MA), which specifically recognizes phosphorylated JNK (Thr²⁴⁷/Tyr³⁸²). After hybridization with primary antibody, the membrane was washed with Tris-buffered saline (TBS) thrice, then incubated with horseradish peroxidase–conjugated secondary antibody (1:5,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature and washed with TBS thrice. Final detection was done with enhanced chemiluminescence reagents. The extent of protein loading was determined by blotting for β-actin. The membrane was incubated in stripping buffer [100 mmol/L β-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl (pH 6.7)] at 50°C for 30 minutes with occasional agitation before incubating in blocking buffer and reprobing with horseradish peroxidase–conjugated secondary antibody (1:5,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature and washed with TBS thrice. Final detection was done with enhanced chemiluminescence reagents. The extent of protein loading was determined by blotting for β-actin. The membrane was incubated in stripping buffer [100 mmol/L β-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl (pH 6.7)] at 50°C for 30 minutes with occasional agitation before incubating in blocking buffer and reprobing with anti–β-actin (Santa Cruz Biotechnology).

**Statistical analyses.** Possible synergistic effects of the combination of TPA and paclitaxel were assessed by the method of model-free tests for synergy (20), which is based on the method of isobologram analysis. Linear interpolations were used for the calculations of the means of the responses for TPA doses alone as needed. The degrees of freedom of the t test statistics in testing for the synergistic effects were determined using Satterthwaite's approximation (21). The analyses of the percentage of initial tumor size were based on the mixed-effect regression (repeated measurement) model (22). The treatment effects were assessed by comparing the rates of change over time between treatment groups (i.e., comparing the slopes between treatment groups).

**Results**

**Effects of TPA or paclitaxel alone or in combination on growth and apoptosis in LNCaP cells.** In our recent studies, we found that treatment of LNCaP cells with TPA resulted in growth inhibition and apoptosis in a concentration-dependent manner (13). In the present study, we assessed the effects of various concentrations of paclitaxel on the growth of LNCaP cells. In these experiments, the cells were seeded in 35-mm tissue culture dishes and incubated for 24 hours to allow the cells time to attach to the culture dishes. The cells were then treated once with ethanol (1 µL/mL) or paclitaxel in ethanol for 96 hours. The number of viable and dead cells was determined by a trypan blue exclusion assay. As shown in Fig. 1, paclitaxel inhibited cell growth and caused cell death in LNCaP cells in a concentration-dependent manner. Treatment of LNCaP cells with 1 to 10 ng paclitaxel/mL for 96 hours resulted in a 15% to 78% decrease in the number of viable cells when compared with control cells treated only with ethanol (Fig. 1A), and 8% to 42% of the cells were dead (Fig. 1B). Ethanol-treated control cells had only 2% cell death (Fig. 1B).

To evaluate the potential synergistic effect of TPA and paclitaxel on the growth of LNCaP cells, we treated these cells with combinations of clinically achievable concentrations of TPA (0.2 ng/mL; ref. 4, 23) and paclitaxel (0.2-2 ng/mL; ref. 24). We found that low concentrations of paclitaxel (0.2-1 ng/mL) or TPA (0.2 ng/mL) alone had only a small inhibitory effect on the growth of LNCaP cells (Fig. 1C). Treatment of LNCaP cells with combinations of TPA (0.2 ng/mL) and paclitaxel (0.2-2 ng/mL) resulted in a strong increase in growth inhibition (Fig. 1C). Analysis of the data for synergy by the isobologram method of Laska et al. (20) revealed synergistic effects for the combinations of TPA (0.2 ng/mL) and paclitaxel (0.2-1 ng/mL). The synergistic effects for the combination of TPA (0.2 ng/mL) and paclitaxel were statistically significant at paclitaxel concentrations of 0.2, 0.5, and 1 ng/mL (P = 0.01, 0.003, and 0.002, respectively). In additional experiments, we found that pretreatment of LNCaP cells with TPA for 48 hours before treatment with paclitaxel also enhanced the inhibitory effect of paclitaxel on the growth of LNCaP cells (data not shown). As shown in Fig. 1D, combinations of TPA (0.2 ng/mL) and paclitaxel (0.2-2 ng/mL) markedly increased the number of dead LNCaP cells. Statistical analysis showed that the synergistic effects for the combination of TPA and paclitaxel were significant at paclitaxel concentrations of 0.2, 0.5, and 1 ng/mL (P = 0.038, 0.008, and 0.004, respectively).

Because the combination of TPA and paclitaxel caused a synergistic decrease in the number of viable LNCaP cells and an increase in dead cells as determined by the trypan blue assay, we used a morphologic assessment to determine whether cell death was by apoptosis. Treatment of LNCaP cells with TPA in combination with paclitaxel resulted in apoptosis in a time-dependent manner (Fig. 1E). Morphologically distinct apoptotic cells from representative samples are shown in Fig. 1H and I. Apoptosis in LNCaP cells induced by TPA alone or in combination with paclitaxel was also determined by flow cytometric analysis. As shown in Table 1, treatment of LNCaP prostate cancer cells with a clinically achievable concentration of TPA (1 ng/mL) or paclitaxel (5 ng/mL) alone for 96 hours resulted in a moderate increase in the percentage of apoptotic cells as measured by determination of pre-G₀-G₁ apoptotic cells by flow cytometry. Treatment of LNCaP cells with a combination of TPA (1 ng/mL) and paclitaxel (5 ng/mL) for 96 hours resulted in 49% apoptotic cells (Table 1). The percentage of apoptotic LNCaP cells after treatment with TPA (1 ng/mL) or paclitaxel (5 ng/mL) alone for 96 hours was 9% and 27%, respectively (Table 1). The number of apoptotic LNCaP cells as determined by morphologic assessment was comparable with that measured by flow cytometric analysis (Table 1). The results indicate that treatment of the cells with a combination of TPA and paclitaxel has a greater than additive effect on apoptosis when compared with the effect of the individual compounds alone.

**Effect of TPA in combination with paclitaxel on the phosphorylation of JNK in LNCaP cells.** Because earlier studies showed that TPA-induced apoptosis in LNCaP cells was associated with activation of JNK (25), we evaluated the phosphorylation of JNK in LNCaP cells treated with TPA alone or in combination with paclitaxel by Western blot analysis. In these experiments, LNCaP cells were treated with TPA (1 ng/mL) or paclitaxel (5 ng/mL) alone or in combination for 0.5, 1, 2, or 4 hours and analyzed by Western blot using an anti–phosphorylated JNK antibody (Cell Signaling Technology). Figure 2 shows a representative result from three separate experiments. Treatment of LNCaP cells with TPA (1 ng/mL) resulted in a small but measurable increase in the level of phosphorylated JNK at 2 hours after the treatment and the level of phosphorylated JNK decreased at 4 hours after the treatment (Fig. 2). Although paclitaxel (5 ng/mL) alone did not induce a detectable change in the level of phosphorylated JNK at any time interval, the combination of TPA (1 ng/mL) and paclitaxel (5 ng/mL) induced a marked increase of phosphorylated JNK and the increase peaked at 2 hours after the treatment. The extent of protein loading was determined by blotting for β-actin, and the
levels of phosphorylated JNK in the Western blot was analyzed by absorbance measurement and normalized for actin. The results showed that the level of phosphorylated JNK in LNCaP cells treated with a combination of TPA (1 ng/mL) and paclitaxel (5 ng/mL) was 10.5- and 3.5-fold higher than that in TPA-treated cells at 2 and 4 hours after the treatment, respectively.

Effects of i.p. injections of TPA or paclitaxel alone or in combination on the growth of LNCaP tumors in immunodeficient mice. In an initial experiment (experiment 1), male NCr mice with well-established tumors (0.65-1 cm long and 0.65-1 cm wide; six mice per group) were injected with vehicle (5 µL/g body weight), TPA (100 ng/g; 5 µL vehicle/g), paclitaxel (10 ng/g; 5 µL vehicle/g), or TPA (100 ng/g) in combination with paclitaxel (10 ng/g) in 5 µL vehicle/g daily for 5 days followed by a 2-day intermission. This protocol of treatment (5 days on and 2 days off) was repeated four times and the experiment was terminated at day 28. Tumor growth was measured and expressed as percentage of initial size (Fig. 3A). Statistical analyses showed that the linear rate of change in percentage tumor size relative to initial size for the vehicle control group was significantly greater than for the TPA-treated group ($P = 0.0005$) and for the TPA + paclitaxel group ($P < 0.0001$), but was not significantly different from the paclitaxel group ($P = 0.2478$). The linear rate of change in percentage tumor size between the TPA and the TPA + paclitaxel groups or between the paclitaxel and the TPA + paclitaxel groups was significantly different ($P = 0.0046$ and 0.0064, respectively). The difference in the linear rate of change in percentage tumor size relative to baseline between the TPA and the paclitaxel groups was not significantly different ($P = 0.8445$). The effect of the various treatments on tumor growth or regression in individual mice (six mice per group) is shown in Fig. 3B and Table 2. None of the animals treated with...
vehicle had tumor regression, 33% of the animals treated with TPA had some tumor regression, 17% of the animals treated with paclitaxel had some tumor regression, and 100% of the animals treated with TPA + paclitaxel for 28 days had some tumor regression (Fig. 3B; Table 2). Statistical analysis of the data in Fig. 3B with the Dunnett’s multiple comparison test showed that the differences in the percentage of initial tumor size at day 28 between the control group and the TPA group and between the control group and the TPA in combination with paclitaxel were statistically significant ($P < 0.05$ and $P < 0.01$, respectively). The effect of the various treatments on body weight is shown in Fig. 4C. The mean ± SE for the percentage of initial body weight (six mice per group) after 28 days of treatment was 93.0 ± 2.3% for the control group, 81.3 ± 2.3% for the TPA group, 84.6 ± 3.3% for the paclitaxel group, and 82.4 ± 4.8% for the TPA + paclitaxel group. Statistical analysis with the Dunnett’s multiple comparison test showed that the differences in the percentage of initial body weight between the control group and any of the treatment groups were not statistically significant ($P > 0.05$).

The difference in percentage of initial tumor size at day 28 in vehicle controls between experiments 1 and 2 was not statistically significant ($P = 0.44$). When treated with TPA alone, the percentage of animals with some tumor regression was 33% in experiment 1 and 100% in experiment 2. The percentage of initial tumor size in TPA-treated animals was significantly smaller in experiment 2 than that in experiment 1 ($P < 0.001$). Although more animals treated with paclitaxel in experiment 2 had tumor regression (57%) compared with that in experiment 1 (17%), there was no statistically significant difference in percentage of initial tumor size between paclitaxel-treated mice in experiments 1 and 2 ($P = 0.398$). All mice treated with TPA in combination with paclitaxel in both experiments had some tumor regression, and the percentage of initial tumor size in mice treated with TPA + paclitaxel was similar in experiments 1 and 2 ($P = 0.077$). The body weight changes of animals in vehicle control groups in the two experiments were similar ($P = 0.102$). The body weight decrease in animals treated with TPA, paclitaxel, or the combination of these two agents as determined by the linear rates of change in percentage of body weight was significantly greater in experiment 2 than that in experiment 1 ($P = 0.007, P < 0.0001$, and $P = 0.017$).

---

**Table 1. Effects of TPA and paclitaxel alone or in combination on apoptosis in LNCaP cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Morphologic assessment</th>
<th>Flow cytometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21 ± 0.3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Ethanol</td>
<td>18 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>TPA (1 ng/mL)</td>
<td>16.6 ± 1.3 (14.8)</td>
<td>11.2 ± 11 (9.3)</td>
</tr>
<tr>
<td>Paclitaxel (5 ng/mL)</td>
<td>26.4 ± 11 (24.6)</td>
<td>28.4 ± 0.7 (26.5)</td>
</tr>
<tr>
<td>TPA (1 ng/mL) + paclitaxel (5 ng/mL)</td>
<td>52.4 ± 1.6 (50.6)</td>
<td>50.8 ± 1.7 (48.9)</td>
</tr>
</tbody>
</table>

**NOTE:** LNCaP cells were seeded at a density of $1 \times 10^5$ cells in 100-mm culture dishes (5 mL/dish) and incubated for 24 hours to allow the cells to attach to the culture dishes. The cells were then treated with TPA (1 ng/mL) and paclitaxel (5 ng/mL) alone or in combination for 96 hours. The final concentration of ethanol vehicle was 1 $\mu$L/mL. Apoptosis was determined by morphologic assessment or flow cytometric analysis as described in Materials and Methods. Each value represents the mean ± S.E from three separate experiments. The values in parentheses have been corrected by subtracting the value for the ethanol-treated control cells.

---

**Fig. 2.** Effect of TPA or paclitaxel alone or in combination on the level of phosphorylated JNK in LNCaP cells. LNCaP cells were seeded at a density of $1 \times 10^5$ cells in 100-mm culture dishes (5 mL/dish) and incubated for 24 hours to allow the cells to attach. The cells were then treated with TPA (1 ng/mL) or paclitaxel (5 ng/mL) alone or in combination for 0.5, 1, 2, or 4 hours. The final concentration of ethanol vehicle was 1 $\mu$L/mL. After treatment, the cells were washed with ice-cold PBS and the cell lysates were analyzed by Western blot with an anti-phospho-JNK antibody as described in Materials and Methods.
Discussion

In the present study, we showed a synergistic inhibitory effect of TPA and paclitaxel on the growth of cultured LNCaP prostate cancer cells. Although treatment of LNCaP cells with a low concentration of TPA or paclitaxel alone had only a modest effect on apoptosis, treatment of the cells with a combination of these two agents resulted in a greater than additive increase in apoptotic cells (Table 1). The concentrations of TPA used to obtain synergistic effects on cell growth (0.2-1 ng/mL) are clinically achievable (4, 23). Our earlier study showed that the peak blood concentrations of TPA ± SD in several patients who received an i.v. infusion of TPA (0.125 mg/m^2) was 1.75 ± 0.55 ng/mL and ranged between 0.3 and 5.2 ng/mL (4, 23). Our recent study showed that the peak blood level of TPA after a 100 ng/g body weight injection was ~1 ng/mL (13). Concentrations of paclitaxel (0.2-5 ng/mL) used in the present study are much lower than the sustainable blood levels of paclitaxel in humans (24). The peak blood level of paclitaxel after treatment of patients with a standard dose of paclitaxel (175 mg/m^2) was >853 ng/mL and the time period that the paclitaxel concentration was >85 ng/mL ranged from 7.6 to 31.6 hours (24). Because treatment with paclitaxel is associated with significant toxic side effects that limit its dose and duration of use (14–18), efforts have been devoted to develop effective combinations of paclitaxel with other anticancer agents. Earlier studies have shown that combinations of paclitaxel and 1α,25-dihydroxyvitamin D3 (26) or paclitaxel and quinacrine (27) enhanced the anticancer activity of paclitaxel. The present study showed that very low concentrations of paclitaxel in combination with TPA synergistically inhibited the growth and increased apoptosis in LNCaP prostate cancer cells. These results suggest that the toxic side effects of paclitaxel may be reduced when a low dose is used in combination with TPA.

In the in vivo studies, we designed two treatment regimens to determine if an intermission decreased the toxicity of TPA and paclitaxel. In experiment 1, NCr mice with LNCaP tumors were treated daily for 5 days followed by a 2-day intermission, which was repeated four times for a total of 28 days. In experiment 2, mice received daily treatment without intermission for 28 days.
Tumor growth and body weight changes were similar in vehicle-treated animals in both experiments. When the animals were treated systemically with TPA (100 ng/g) or paclitaxel (10 ng/g) alone, a greater inhibitory effect on tumor growth was found in experiment 2, but the toxicity as reflected by body weight loss was also increased in experiment 2. The number of animals that had some tumor regression and the percentage of initial tumor size in mice treated with TPA + paclitaxel was similar in experiments 1 and 2. Although the body weight decrease in animals treated with TPA + paclitaxel was greater in experiment 2 than in experiment 1, the differences in body weight change between vehicle control and TPA + paclitaxel group in both experiments were not statistically significant (P > 0.05). Results from the present study indicate that continuous daily treatment with TPA or paclitaxel alone had a greater inhibitory effect on tumor growth than daily treatment for 5 days followed by a 2-day intermission. However, the effect of TPA + paclitaxel on tumor growth was similar in both experiments. Although all mice treated with TPA alone or TPA in combination with paclitaxel in experiment 2 had tumor regression, the tumor size in animals treated with a combination of TPA and paclitaxel was significantly smaller than that of animals treated with TPA alone.

The mechanism of TPA-induced apoptosis in prostate cancer cells is largely unknown. An earlier study showed that TPA-induced apoptosis in LNCaP cells was associated with translocation of protein kinase Ca (PKCa) to nonnuclear membranes implying activation of this enzyme, and TPA-resistant LNCaP cells had down-regulation of PKCa (9). Other studies provided evidence that TPA-induced apoptosis in LNCaP cells is mediated via PKCα and that overexpression of this isoform of PKC resulted in increased sensitivity of LNCaP cells to TPA-induced apoptosis (10). The signal transduction pathway downstream of TPA-activated PKC that leads to apoptosis is not well known. A close correlation between PKC activation, ceramide elevation, and apoptosis in response to TPA suggests that ceramide may play an important role in a pathway by which TPA-activated PKC induces apoptosis in LNCaP cells (7). TPA-induced activation of PKC may also lead to the translocation of a nuclear hormone orphan receptor, TR3/Nur77, from the nucleus to the mitochondria with induction of cytochrome c release and subsequent apoptosis (28). In addition, it was reported that TPA induced phosphorylation of JNK in LNCaP cells (29) and activation of JNK is required for TPA-induced apoptosis in LNCaP cells (25). The results of the present study showed that TPA (1 ng/mL) alone caused a small increase in phosphorylated JNK in LNCaP cells and that paclitaxel (1 ng/mL) was inactive. However, a combination of TPA and paclitaxel caused a strong increase in the level of phosphorylated JNK. Taken together, our studies as well as those by other investigators suggest that JNK may be involved in TPA-induced apoptosis in LNCaP cells.

Paclitaxel binds to microtubules and causes kinetic suppression of microtubule dynamics (30). The consequent arrest of the cell cycle in mitosis has been considered the cause of paclitaxel-induced cytotoxicity (31). However, biochemical events downstream of paclitaxel binding to microtubules that lead to apoptosis are not well understood. Recent discoveries of activation of signaling molecules by paclitaxel and paclitaxel-initiated transcriptional activation of various genes indicate that paclitaxel initiates apoptosis through multiple mechanisms (32). One of the factors that is involved in paclitaxel-induced cancer cell death is JNK. High dose levels of paclitaxel were shown to induce activation of JNK in a dose-dependent manner (33, 34). In an earlier study, Wang et al. (33) showed that paclitaxel at 8.5 ng/mL had little effect on activation of JNK but higher concentrations of paclitaxel (85-8,530 ng/mL) induced 3- to 8-fold increases in JNK activation. As indicated above, paclitaxel (5 ng/mL) alone did not increase phosphorylation of JNK in the present study but when paclitaxel at this low concentration was combined with TPA, phosphorylation of JNK increased significantly.
clinically relevant concentration was combined with a low clinically relevant concentration of TPA, a marked increase in the phosphorylation of JNK in LNCaP cells was observed. The mechanism by which TPA in combination with paclitaxel induced a synergistic increase in growth inhibition and cell death in LNCaP cells is not known. Our study showed that a combination of TPA and paclitaxel markedly increased the phosphorylation of JNK in this cell line, and TPA and paclitaxel may activate JNK through different mechanisms. Activation of JNK in LNCaP cells by TPA is mediated by activation of PKC (29). Paclitaxel induces activation of JNK through signal transduction via both Ras and apoptosis signal-regulating kinase 1 (ASK1; ref. 33). Although a low concentration of TPA (1 ng/mL) only had a small effect on the phosphorylation of JNK and paclitaxel (5 ng/mL) did not cause a measurable increase in the level of phosphorylated JNK, a combination of TPA (1 ng/mL) and paclitaxel (5 ng/mL) markedly increased the level of phosphorylated JNK. This finding indicates that a combination of agents that have different mechanisms of action may produce a synergistic effect on the activation of JNK. The synergistic activation of JNK may be one of the mechanisms by which TPA in combination with paclitaxel induces a substantial increase in LNCaP cell killing. Both TPA and paclitaxel have been shown to induce manganese superoxide dismutase (35). Whether TPA in combination with paclitaxel causes a synergistic induction of this enzyme in prostate cancer cells to enhance apoptosis needs further studies.

In summary, the present study showed that TPA in combination with paclitaxel synergistically inhibited the growth and induced cell death in human prostate cancer LNCaP cells. The concentrations of TPA and paclitaxel required for these effects are clinically achievable. Treatment of immunodeficient mice with LNCaP tumors with a combination of TPA and paclitaxel resulted in tumor regression. Clinical studies with TPA alone or in combination with paclitaxel in patients with prostate cancer may be warranted.

Acknowledgments

We thank Florence Florek for her excellent help in the preparation of the manuscript.

References

Effects of 12-O-Tetradecanoylphorbol-13-acetate (TPA) in Combination with Paclitaxel (Taxol) on Prostate Cancer LNCaP Cells Cultured In vitro or Grown as Xenograft Tumors in Immunodeficient Mice

Xi Zheng, Richard L. Chang, Xiao-Xing Cui, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/11/3444

Cited articles
This article cites 33 articles, 21 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/11/3444.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/12/11/3444.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/12/11/3444.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.