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Inhibitory Effect of 12-*O*-Tetradecanoylphorbol-13-acetate Alone or in Combination with All-*trans*-Retinoic Acid on the Growth of LNCaP Prostate Tumors in Immunodeficient Mice

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ABSTRACT

Clinically achievable concentrations of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 0.16–0.32 nM) and all-*trans*-retinoic acid (ATRA; 0.5–1 μM) had a synergistic inhibitory effect on the growth of cultured LNCaP prostate cancer cells, and apoptosis was markedly stimulated. In additional studies, NCr immunodeficient mice received s.c. injection with LNCaP cells in Matrigel. After 4–6 weeks, mice with well-established tumors received i.p. injection with vehicle, TPA (0.16 nmol/g body weight), ATRA (0.5 nmol/g body weight), or TPA+ATRA in vehicle once a day for 46 days. Tumor growth occurred in all of the vehicle-treated control mice. The percentage of animals with some tumor regression after 21 days of treatment was 0% for the control group, 31% for the ATRA group, 62% for the TPA group, and 100% for the TPA+ATRA group (13 mice/group). Although treatment of the mice with TPA or TPA+ATRA continued to inhibit tumor growth for the duration of the 46-day study, treatment of the mice with ATRA alone did not inhibit tumor growth beyond 28 days of daily injections (6 mice/group). Mechanistic studies indicated that treatment of the mice with TPA or TPA+ATRA for 46 days increased apoptosis in the tumors, and treatment with TPA+ATRA also decreased the mitotic index. Because the dose of TPA used in this study was effective and resulted in clinically achievable blood levels, clinical trials with TPA alone or in combination with ATRA in patients with prostate cancer may be warranted.

INTRODUCTION

Croton tiglium L is a leafy shrub of the Euphorbiaceae family that is native to Southeastern Asia. The seed oil (croton oil) obtained from this plant or its major active constituent, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is an irritant and inflammatory agent that has been used widely as a tumor promoter (usual dose = 5–16 nmol, twice a week) on the skin of mice previously initiated with 7,12-dimethylbenz(*a*)anthracene or other polycyclic aromatic hydrocarbons (1–6). Topical application of 5–16 nmol of TPA alone to mouse skin twice a week for several months either has no tumorigenic effect by itself or results in only an occasional nonmalignant papilloma. A rough estimate of the concentration of TPA in the epidermis after each application is 10,000–32,000 nM. TPA at a 10,000-fold lower concentration is an extraordinarily potent stimulator of differentiation in myeloid leukemia cells *in vitro* (7–10). Because of the potent effect of TPA in stimulating differentiation in myeloid leukemia cells at a very low concentration, we studied the effects of TPA in patients with myeloid leukemia. In a preliminary study, our laboratory, together with colleagues in China, demonstrated pharmacological activity for i.v. administered 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 peripheral blood were observed as well as temporary remission of disease symptoms without serious toxicity (11). In an additional study, i.v. infusions of TPA increased the number of circulating WBCs and neutrophils in patients with depressed bone marrow caused by prior treatment with cytotoxic chemotherapeutic drugs (12). The results obtained in both studies and preliminary data from a Phase I trial with TPA (13) at the Cancer Institute of New Jersey (New Brunswick, NJ) indicated an acceptable toxicity profile. The Phase I trial on the effects of TPA in myeloid leukemia patients is continuing. In an additional study, a low, clinically achievable concentration of TPA (0.16 nM) in combination with clinically achievable concentrations of all-*trans*-retinoic acid (ATRA; 0.1–1 μM), 1α,25-dihydroxyvitamin D_3 (1 nM), or sodium butyrate (100 μM) synergistically inhibited growth and stimulated 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 (14).

In studies with solid tumors, TPA was shown to inhibit the growth, stimulate apoptosis, or enhance differentiation in human tumor cell lines derived from patients with melanoma or prostate, breast, colon, or lung cancer (15–19). Treatment of prostate cancer LNCaP cells with clinically achievable concentrations of TPA (1–1.6 nM) resulted in growth inhibition (15, 20–22), and treatment of these cells with a severalfold higher concentration of TPA caused apoptosis (15, 20–22). Treatment of LNCaP cells with a combination of TPA and γ-radiation resulted in a synergistic increase in ceramide synthesis and apoptosis (23). In other studies, treatment of prostate cancer cells with ATRA or 9-*cis*-retinoic acid inhibited growth and induced apoptosis (24, 25). Because of these studies on the effects of TPA and ATRA on growth and apoptosis in cultured prostate cancer cells and the acceptable toxicity profile of TPA in humans, we have initiated studies on the effects of TPA alone and in combination with ATRA *in vitro* on the growth of cultured LNCaP prostate cancer cells and *in vivo* in immunodeficient mice. In the present report, we describe a synergistic inhibitory effect of TPA and ATRA on the growth of cultured prostate cancer LNCaP cells, and we also describe an inhibitory effect of TPA or ATRA administration on the growth of well-established LNCaP tumors in immunodeficient mice. Tumor regressions were observed in several of the treated mice, and administration of a combination of TPA and ATRA to these tumor-bearing mice resulted in some tumor regression in all of the treated animals. These are the first studies to indicate an inhibitory effect of *in vivo* administration of TPA on the growth of tumors in animals.

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MATERIALS AND METHODS

Cell Culture and Reagents. LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA). TPA was obtained from Alexis Co. (San Diego, CA). ATRA, propylene glycol, polysorbate 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, L-glutamine, and fetal bovine serum were from Gibco (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 L-glutamine (300 μg/ml) as described previously (14). Cultured cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and passaged twice a week. In all experiments, LNCaP cells were initially seeded at a density of 1×10^5 cells/ml in a final volume of 2 or 5 ml in 35- or 60-mm 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 hemocytometer under a light microscope (Nikon Optiphot). 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 min. Blue cells were counted as dead cells, and the cells that did not absorb dye were counted as live cells.

Flow Cytometry. To perform the analysis, 1×10^6 cells were washed with PBS and resuspended in 500 μl of stain solution (20 mg/ml polyethylene glycol 8000, 50 μg/ml propidium iodide, 0.1% Triton X-100, and 0.4 M 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 G_1 phase, where they have two copies of their genome (2N), the G_2-M phase, where they have four copies of their genome (4N), or 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-G_0-G_1 cells were considered apoptotic cells. The proportion of cells in each phase of the cell cycle was calculated by using cytologic software from Coulter Corp.

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 filter-capped microisolator cages and provided with sterilized food and water. Prostate cancer LNCaP cells (2×10^6 cells/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 4–6 weeks, mice with well-established tumors (0.65–1.0-cm long and 0.65–1.0-cm wide) were used for the various experiments. In all experiments, 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 $\times$ width) and body weight were measured daily. At the end of the study, mice were sacrificed, and tumors were excised, weighed, placed in phosphate-buffered formalin at room temperature for 48 h, and then placed in ethanol for 48 h before preparing paraffin sections as described previously (26). All animal experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol.

Blood Levels of TPA. The concentration of TPA in blood after an i.p. injection of TPA was measured by a recently developed bioassay that quantifies ethyl acetate-extractable differentiating activity in blood (27). Differentiating activity was measured in HL-60 cells and expressed as nanogram equivalents of TPA. Anticipated de-esterified metabolites of TPA (phorbol, phorbol-13-acetate, or phorbol-12-myristate) do not interfere with the assay because they are not active in the bioassay. Although we express the blood levels of bioactive material as ng TPA/ml blood, we realize that the assay could also include biologically active metabolites. Attempts at developing a mass spectrometry assay for TPA were not successful.

Caspase 3 (Active Form) Immunostaining. Affinity-purified polyclonal rabbit antibody that reacts with the p17 subunit of human and mouse caspase 3 but does not react with the precursor form was purchased from R&D Systems (catalogue number AF835). Tumor sections used for the measurement of caspase 3 (active form) were stained by the horseradish peroxidase-conjugated avidin method, with some modification. Endogenous peroxidase was blocked by incubating the tissue sections in 3% hydrogen peroxide in methanol for 30 min at room temperature. Sections were then treated with 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven at high temperature for 10 min. The sections were incubated with a protein block (normal goat serum) for 10 min, followed by avidin D for 15 min and biotin blocking solution for 15 min (avidin-biotin blocking kit from Vector Laboratories) at room temperature. The sections were incubated with caspase 3 primary antibody (1:2000 dilution) for 30 min at room temperature followed by incubation with a biotinylated antirabbit secondary antibody for 30 min and incubation with conjugated avidin solution (ABC Elite kit purchased from Vector Laboratories) for 30 min. Color development was achieved by incubation with 0.02% 3,3′-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide for 10 min at room temperature. The slides were then counterstained with hematoxylin and dehydrated, and coverslips were added for permanent mounting. A positive reaction was shown as a light brown to dark brown precipitate in the cytoplasm and/or perinuclei of the cells. The percentage of caspase 3-positive cells was determined in each tumor.

Bromodeoxyuridine (BrdUrd) Incorporation into DNA. BrdUrd, a thymidine analog that is incorporated into proliferating cells during S phase, is detected by a biotinylated monoclonal anti-BrdUrd antibody and visualized by using streptavidin-peroxidase and 3,3′-diaminobenzidine, which stains BrdUrd-containing nuclei a dark brown (staining kit from Oncogene Research Products, Cambridge, MA). Briefly, all animals received i.p. injection with BrdUrd (50 mg/kg) and were killed 1 h later. Endogenous peroxidase was blocked by incubating the tissue sections in 3% hydrogen peroxide in methanol for 10 min at room temperature. The tissue sections were then incubated in a moist chamber with 0.125% trypsin for 10 min at 37°C, rinsed in deionized water, and incubated at room temperature for 30 min with denaturing solution (Oncogene Research Products). The sections were incubated with blocking solution for 10 min at room temperature and covered with biotinylated mouse monoclonal anti-BrdUrd antibody (Oncogene Research Products) at room temperature for 90 min. Sections were rinsed with PBS and incubated with streptavidin-peroxidase for 10 min. Color development was achieved by incubation for 5 min at room temperature with a substrate solution containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide. The slides were weakly counterstained with Mayer's hematoxylin (Sigma) for 2 min, cleared with xylene, mounted with a coverslip, and scored under a light microscope. The BrdUrd-positive cells were expressed as the percentage of positive cells.

Statistical Analyses. The possible synergistic effects of the combination of TPA and ATRA were assessed by the method of model-free tests for synergy (28), 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 (29). The analyses of the percentage of initial tumor size were based on the mixed effect regression (repeated measurement) model (30). The treatment effects were assessed by comparing the rates of change over time between treatment groups (*i.e.*, comparing the slopes between treatment groups). Pearson correlation coefficients together with 95% confidence intervals were calculated to assess the strength of correlations between tumor weight and tumor size and between percentage of initial tumor size and ratio of percentage of mitotic cells to caspase 3-positive cells. For the correlation between percentage change in tumor size from baseline and percentage change in body weight from baseline at day 21 and at day 46, tests of correlation $\rho = 0$ *versus* $\rho \neq 0$ were performed first. If a correlation was significantly different from 0, a 95% confidence interval was provided.

RESULTS

Effects of TPA Alone or in Combination with ATRA on Growth and Apoptosis in Cultured LNCaP Prostate Cancer Cells

In initial studies, we assessed the effects of various concentrations of TPA on the growth of LNCaP cells. In these experiments, the cells were seeded in 35-mm tissue culture dishes and incubated for 24 h to allow the cells to attach to the culture dishes. The cells were then treated once with ethanol (2 μl/ml) or TPA in ethanol for 96 h. The number of viable and dead cells was determined by trypan blue exclusion. As shown in Fig. 1, treatment with TPA inhibited cell

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growth and caused cell death in a concentration-dependent manner. Treatment of LNCaP cells with 0.2–10 ng TPA/ml (0.32–16 nM) for 96 h resulted in an 18–91% decrease in the number of viable cells when compared with control cells treated only with ethanol (Fig. 1*A*), and 18–83% of the cells were dead (Fig. 1*B*). Ethanol-treated control cells had only 2–4% cell death (Fig. 1*B*). Treatment of LNCaP cells with 3.2 nM TPA resulted in morphologically distinct apoptotic cells (Fig. 2, *A* and *B*). The dose response for TPA-induced increases in morphologically distinct apoptotic LNCaP cells is shown in Fig. 2*C*. These results using TPA-induced morphological changes are similar to those for TPA-induced increases in dead cells as measured with trypan blue (Fig. 1*B*). The results from multiple experiments in our laboratory indicated that the concentration of TPA that caused a 50%

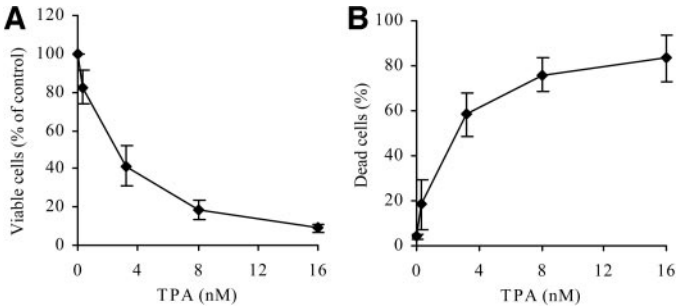

Fig. 1. Effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on the growth and death of LNCaP cells. LNCaP cells were seeded at a density of 1×10^5 cells/ml in 35-mm tissue culture dishes (2 ml/dish) and incubated for 24 h. The cells were then treated once with ethanol (2 μl/ml) or with various concentrations of TPA (0.32–16 nM) in ethanol for 96 h. Each incubation mixture had 2 μl ethanol/ml except for the untreated control. *A*, number of viable cells after treatment with TPA is expressed as percentage of untreated control. *B*, the percentage of dead cells was determined by a trypan blue exclusion assay. Each value is the mean ± SE from three separate experiments.

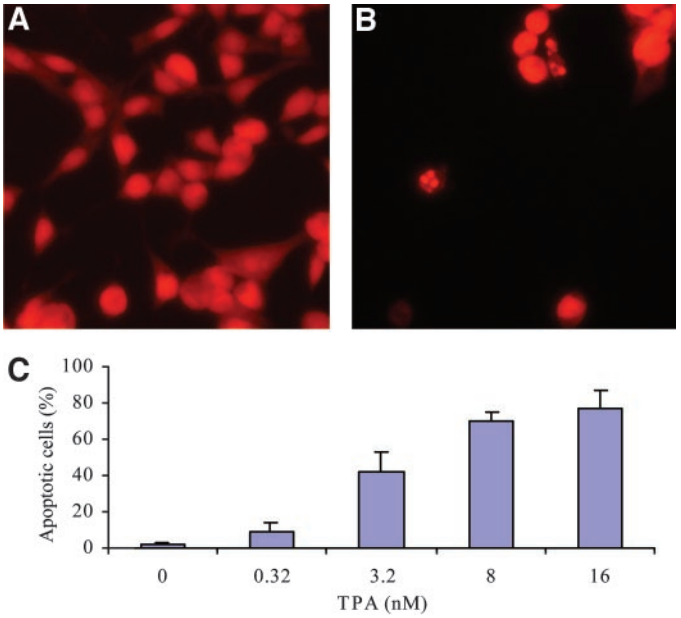

Fig. 2. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-induced apoptosis in LNCaP cells. LNCaP cells were seeded at a density of 1×10^5 cells/ml in 35-mm tissue culture dishes and incubated for 24 h. The cells were then treated once with ethanol (2 μl/ml) or with TPA (3.2 nM) in ethanol for 96 h. The final concentration of ethanol was 2 μl/ml in both samples. The cells were fixed with methanol/acetone (1:1) for 10 min and stained with propidium iodide (1 μg/ml) for 10 min. Apoptotic cells were determined by morphological assessment using a fluorescence microscope. *A*, control LNCaP cells. *B*, LNCaP cells treated with 3.2 nM TPA for 96 h. Apoptotic cells with condensed and fragmented nuclei are shown. *C*, percentage of apoptotic cells after treatment with TPA was determined by morphological assessment using a fluorescence microscope (Nikon Eclipse TE200). Each value is the mean ± SE from three separate experiments.

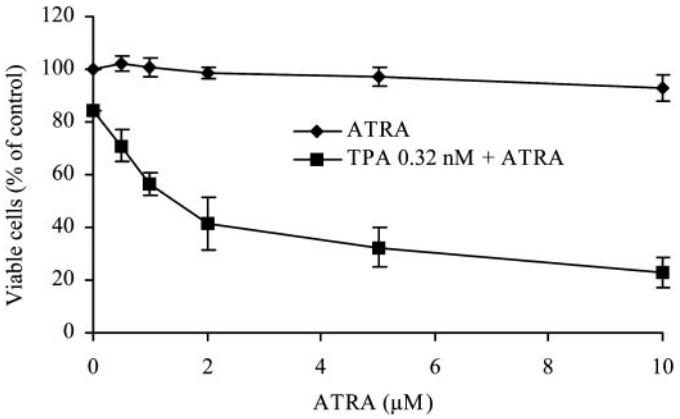

Fig. 3. Effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) alone or in combination with all-*trans*-retinoic acid (ATRA) on the growth of LNCaP cells. LNCaP cells were seeded at a density of 1×10^5 cells/ml in 35-mm tissue culture dishes (2 ml/dish) and incubated for 24 h. The cells were then treated once with various concentrations of ATRA (1–10 μM) alone or in combination with 0.32 nM TPA for 96 h. ATRA and TPA were added in ethanol (final concentration was 2 μl/ml in all samples). The number of viable cells after treatment with ATRA alone or in combination with TPA was determined by a trypan blue exclusion assay. Each value is the mean ± SE from three separate experiments.

inhibition of LNCaP cell growth ranged from 1 to 3 nM (data not presented). Because the maximum blood level of TPA achieved in patients who received a 1-h infusion of TPA (0.125 mg/m^2; ~0.25 mg) ranged from 0.5 to 8.4 nM (27), our results indicate that clinically achievable levels of TPA had a strong inhibitory effect on the proliferation and viability of LNCaP cells, and apoptosis was increased.

To evaluate the potential synergistic effect of TPA and ATRA on the growth of LNCaP cells, we determined the effects of TPA and ATRA alone (Figs. 1 and 3) and in combination on the growth of LNCaP cells (Fig. 3). We found that ATRA (0.5–10 μM) had little or no effect on the growth of LNCaP cells and that TPA (0.32 nM) had a small inhibitory effect on the growth of these cells (Fig. 3). Increasing the concentration of ATRA in the presence of 0.32 nM TPA resulted in a substantial increase in growth inhibition (Fig. 3). Analysis of the data for synergy by the isobologram method of Laska *et al.* (28) revealed synergistic effects for the combination of TPA (0.32 nM) and various concentrations of ATRA. The synergistic effects for the combination of TPA (0.32 nM) and ATRA were statistically significant at ATRA concentrations of 1, 2, and 5 μM ($P = 0.002$, 0.001, and <0.001, respectively) and marginally significant at an ATRA concentration of 0.5 μM ($P = 0.068$).

Although treatment of LNCaP cells with 0.32 nM TPA or 1 μM ATRA alone for 96 h had little or no effect on apoptosis, treatment of these cells with a combination of TPA and ATRA resulted in a marked stimulation of apoptosis as measured by the percentage of pre-G_0-G_1 cells using flow cytometry (Table 1). Untreated or ethanol-treated control cells incubated for 96 h had 2.6% apoptotic cells, and cells treated with TPA (0.32 nM) or ATRA (1 μM) had 7.0% and 3.1% apoptotic cells, respectively. In contrast to the lack of an apoptotic effect of low concentrations of TPA or ATRA alone on apoptosis in LNCaP cells, treatment of LNCaP cells with 0.32 nM TPA and 1 μM ATRA resulted in 56% apoptotic cells (Table 1).

Effect of TPA Alone or in Combination with ATRA on the Growth of Cultured C4-2 Prostate Cancer Cells

In additional studies, we evaluated the effect of TPA alone or in combination with ATRA on the growth of the prostate cancer C4-2 cell line, a variant of the LNCaP cell line that is androgen independent (31). Treatment of C4-2 cells with TPA (0.32 nM) for 96 h decreased

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Table 1 *Effects of TPA[a] alone or in combination with ATRA on proliferation, cell cycle distribution, and apoptosis in cultured LNCaP cells*

LNCaP cells were seeded at a density of 1.0×10^5 cells/ml medium in 60-mm culture dishes (5 ml/dish) and incubated for 24 h. The cells were then treated with TPA (0.32 nM) or ATRA (1 μM) alone or in combination with TPA in fresh medium at 0, 24, 48, and 72 h. All samples except the untreated control received the same amount of ethanol (2 μl/ml). The number of viable cells, cell cycle distribution, and apoptotic cells were measured 96 h after the first treatment. Each value represents the mean ± SE from three experiments.

Treatment	No. of viable cells (1×10^5)	Percentage of cells in			Percentage of apoptotic cells
		G_0-G_1	S	G_2-M	
Untreated control	10.9 ± 0.59	71.4 ± 1.4	16.4 ± 0.7	9.4 ± 0.6	2.6 ± 0.5
Ethanol	10.8 ± 0.57	70.5 ± 1.5	17.5 ± 1.0	9.3 ± 0.4	2.6 ± 0.7
TPA (0.32 nM)	7.6 ± 0.45	68.4 ± 1.7	15.8 ± 0.9	8.6 ± 0.4	7.0 ± 0.9
ATRA (1 μM)	10.3 ± 0.44	72.4 ± 0.5	15.7 ± 0.7	8.5 ± 0.3	3.1 ± 0.5
TPA (0.32 nM) + ATRA (1 μM)	2.5 ± 0.71	32.4 ± 1.8	6.9 ± 1.5	4.2 ± 0.5	56.3 ± 3.4

[a] TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ATRA, all-*trans*-retinoic acid.

Table 2 *Effect of TPA[a] alone or in combination with ATRA to inhibit the growth and to enhance cell killing in C4-2 cells*

C4-2 cells were seeded at a density of 1.0×10^5 cells/ml medium in 35-mm culture dishes (2 ml/dish) and incubated for 24 h. The cells were then treated with TPA (0.32 nM) alone or in combination with ATRA (1–5 μM) for 96 h. The number of viable and dead cells was determined at 96 h by using a trypan blue exclusion assay.

Treatment	No. of viable cells/ml (1×10^5)	Percentage decrease of viable cells	Percentage of dead cells
Experiment 1			
Untreated control	10.8		1.6
Ethanol	10.8		1.6
TPA (0.32 nM)	8.9	17.6	15.8
ATRA (1 μM)	10.7	1.0	2.4
TPA (0.32 nM) + ATRA (1 μM)	6.1	43.5	31.0
Experiment 2			
Untreated control	10.3		1.5
Ethanol	10.2		1.6
TPA (0.32 nM)	8.3	18.7	12.3
ATRA (1 μM)	10.2		1.7
ATRA (2 μM)	10.0	2.0	2.1
ATRA (5 μM)	9.7	5.0	3.6
TPA (0.32 nM) + ATRA (1 μM)	6.3	38.3	28.6
TPA (0.32 nM) + ATRA (2 μM)	5.9	42.2	35.8
TPA (0.32 nM) + ATRA (5 μM)	4.1	59.8	41.4

TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ATRA, all-*trans*-retinoic acid.

the number of viable cells by 18% when compared with control cells that were untreated or treated with vehicle. Treatment of C4-2 cells with ATRA (1 μM) alone for 96 h was inactive, but treatment of the cells with a combination of TPA (0.32 nM) and ATRA (1 μM) for 96 h decreased the number of viable cells by 44%, and similar results were obtained in a second experiment (Table 2). Although a formal isobologram analysis was not done, the results suggest that TPA and ATRA have a synergistic effect on the growth of C4-2 cells. The inhibitory effect of these treatments on cell growth was paralleled by increased cell death (Table 2).

Blood Levels of TPA after Its i.p. Injection in NCr Mice

An i.p. injection of 100 or 200 ng TPA/g body weight in a vehicle that consisted of propylene glycol, polysorbate 80, benzyl alcohol, ethanol, and water (40:0.5:1:10:48.5) resulted in rapid systemic absorption of TPA, with peak blood levels occurring within 30–60 min (Fig. 4). The peak blood level of TPA after a 200 ng/g body weight injection was about 3 ng/ml (4.8 nM), and after a 100 ng/g injection, the peak blood level of TPA was about 1 ng/ml (1.6 nM).[6] The half-life of TPA in these mice (measured between 3 and 8 h after the dose) was about 3–6 h. No detectable TPA was observed at 8, 12, or 24 h after an i.p. injection of 100 ng TPA/g body weight. In an additional study, the i.p. injection of 100 ng TPA/g body weight once a day for 3 weeks gave a similar TPA blood level profile as that observed after a single 100 ng/g i.p. injection of TPA, and negligible blood levels were observed at 24 h after the last dose (data not presented). These results indicate that daily i.p. injections of TPA for 3 weeks did not inhibit or stimulate its own metabolism. The results of these studies indicated good absorption of TPA after an i.p. injection, and they also indicated the feasibility of additional studies to determine the effects of i.p. injections of TPA on the growth of human prostate cancer xenografts in immunodeficient mice.

Effects of i.p. Injections of TPA on the Growth of LNCaP Prostate Tumors in Immunodeficient Mice

Effects of i.p. Injections of TPA or ATRA Alone or in Combination Once a Day for 21 Days on the Growth of LNCaP Tumors in Immunodeficient Mice. Male NCr mice with well-established LNCaP tumors (0.65–1 cm long and 0.65–1 cm wide) received injection with vehicle (5 μl/g body weight), TPA (0.16 nmol/g; 5 μl vehicle/g), ATRA (0.5 nmol/g; 5 μl vehicle/g), or TPA (0.16 nmol/g) in combination with ATRA (0.5 nmol/g) in 5 μl vehicle/g once a day for 21 days (13 mice/group), and tumor growth was measured and expressed as percentage of initial size (Fig. 5*A*). The rates of change in percentage of initial tumor size were significantly different between any two groups ($P = 0.014$ for the test between ATRA and TPA groups, and $P < 0.001$ for tests between any of the other two groups). The mean ± SE for percentage of initial tumor size was 154.2 ± 10.2 for the control group, 96.4 ± 3.1 for the TPA group, 109 ± 5.5 for the ATRA group, and 67.6 ± 4.8 for the TPA+ATRA group (Fig. 5*B*). The effect of the various treatments on tumor growth or regression in individual mice (13 mice/group) is shown in Fig. 5*B* and Table 3. None of the animals treated with vehicle had tumor regression, 31% of the animals treated with ATRA had some tumor regression, 62% of

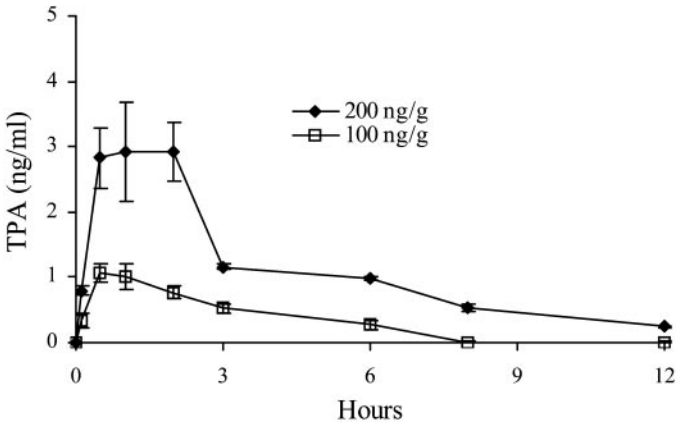

Fig. 4. Blood levels of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in NCr mice after an i.p. injection of TPA. Male NCr mice received an i.p. injection of 100 ng (0.16 nmol) or 200 ng (0.32 nmol) of TPA per g body weight in vehicle (5 μl/g) as described in "Materials and Methods." The mice were sacrificed at 0.08, 0.5, 1, 2, 3, 6, 8, and 12 h after the injection, and blood samples were collected and analyzed for TPA using a recently developed bioassay (27). Each value is the mean ± SE from three animals for the 100 ng/g dose and two to three animals for the 200 ng/g dose.

[6] Blood level data are expressed as nanogram equivalents of TPA per milliliter of blood as measured by the bioassay described in "Materials and Methods."

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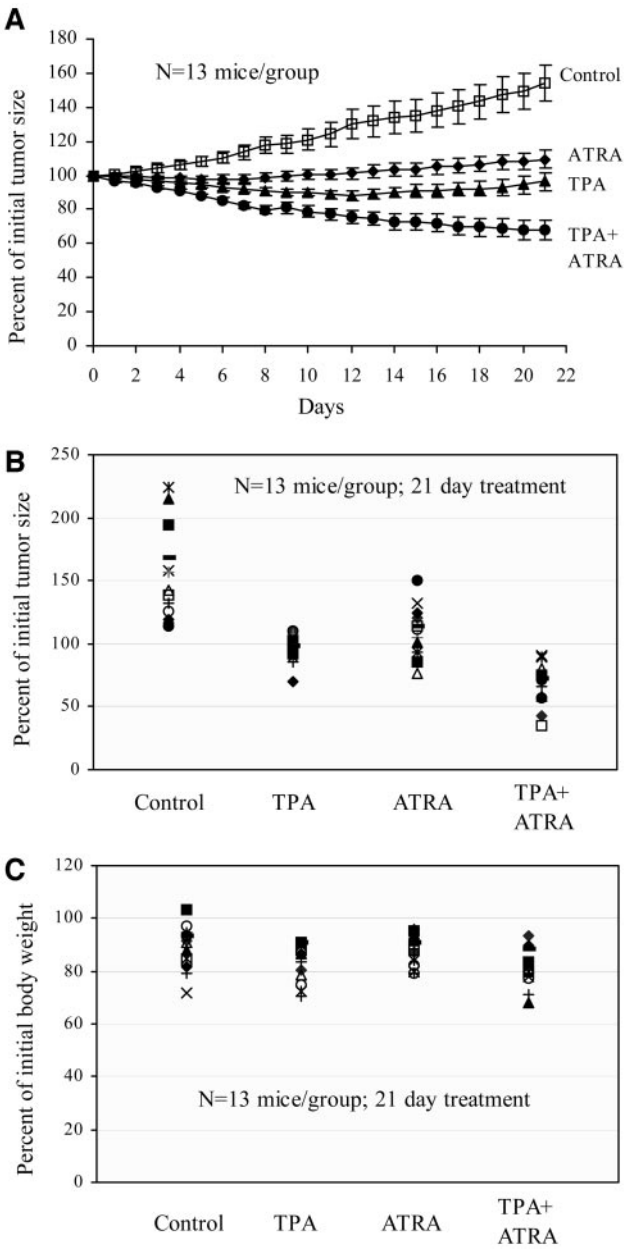

Fig. 5. Effects of i.p. injections of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or all-*trans*-retinoic acid (ATRA) alone or in combination for 21 days on the growth of LNCaP tumors and body weight of NCr immunodeficient mice. Male NCr mice with LNCaP tumors (0.65–1 cm long and 0.65–1 cm wide) were randomly assigned to four groups (13 mice/group). Animals in group 1 received i.p. injections of vehicle (5 μl/g body weight), animals in group 2 received i.p. injections of TPA (0.16 nmol/g; 5 μl vehicle/g), animals in group 3 received i.p. injections of ATRA (0.5 nmol/g; 5 μl vehicle/g), and animals in group 4 received i.p. injections of TPA (0.16 nmol/g) in combination with ATRA (0.5 nmol/g) in 5 μl vehicle/g once a day for 21 days. The composition of the vehicle is described in "Materials and Methods." Tumor size (length $\times$ width) was measured and expressed as percentage of initial size. *A*, growth curve of LNCaP tumors in each group. Each value represents the mean $\pm$ SE. *B*, change in individual tumor size after treatment for 21 days. The mean $\pm$ SE for percentage of initial tumor size was 154.2 $\pm$ 10.2 for the control group, 96.4 $\pm$ 3.1 for the TPA group, 109 $\pm$ 5.5 for the ATRA group, and 67.6 $\pm$ 4.8 for the TPA and ATRA group. *C*, change in individual body weight of mice after treatment for 21 days. The mean $\pm$ SE for percentage of initial body weight of the mice was 88.7 $\pm$ 2.3% for the control group, 83.0 $\pm$ 1.7% for the TPA group, 87.4 $\pm$ 2.0% for the ATRA group, and 80.8 $\pm$ 1.9% for the TPA and ATRA group.

the animals treated with TPA had some tumor regression, and all of the animals treated with TPA+ATRA for 21 days had some tumor regression (Fig. 5*B* and Table 3).

The effect of the various treatments on body weight is shown in Fig. 5*C*. The mean $\pm$ SE for the percentage of initial body weight (13 mice/group) was 88.7 $\pm$ 2.3% for the vehicle-treated control group, 83 $\pm$ 1.7% for the TPA group, 87.4 $\pm$ 2.0% for the ATRA group, and 80.8 $\pm$ 1.9% for the TPA+ATRA group. Statistical analysis with the Dunnett multiple comparison test showed that the differences in the percentage of initial body weight between the control group and the ATRA- or TPA-treated groups were not statistically significant ($P > 0.05$). A statistically significant difference was observed in the percentage of initial body weight between the control group and the ATRA+TPA group ($P < 0.05$). A comparison of data relating changes in body weight to changes in tumor size in individual mice from all 52 animals treated for 21 days indicated a poor relationship between these two parameters ($r = 0.225$). There was no statistically significant relationship between these two parameters in the 26 mice treated with TPA alone or in combination with ATRA for 21 days ($r = -0.06$).

Effects of i.p. Injections of TPA or ATRA Alone or in Combination Once a Day for 46 Days on the Growth of LNCaP Tumors in Immunodeficient Mice. Subgroups of 6 mice/group from the animals described in Fig. 5*A* were continued on the same treatments for an additional 25 days (total of 46 days of treatment), and these mice were analyzed separately (Fig. 6; Table 3). Although the rate of tumor growth for the six animals in each of the four groups described in Fig. 6*A* was similar to that described in Fig. 5*A* for the first 21 days of treatment, at later time intervals, tumors in the ATRA group grew at about the same rate as tumors in the vehicle-treated control animals (Fig. 6*A*). Tumors in the TPA or TPA+ATRA group at these later time intervals grew more slowly than tumors in the vehicle-treated control mice (Fig. 6*A*). The mean $\pm$ SE for percentage of initial tumor size for the entire 46-day treatment interval (6 mice/group) was 211.1 $\pm$ 35.2% for the control group, 106.1 $\pm$ 21.0% for the TPA group, 159.2 $\pm$ 27.0% for the ATRA group, and 78.2 $\pm$ 11.3% for the TPA+ATRA group (Fig. 6*B*). Statistical analysis of the data in Fig. 6*B* with the Dunnett multiple comparison test showed that the differences in the percentage of initial tumor size between the control group and the TPA group and between the control group and the TPA+ATRA group were statistically significant ($P < 0.05$ and $P < 0.01$, respectively). The effect of the various treatments on tumor growth or regression in individual mice (6 mice/group) during the 46-day treatment period is shown in Fig. 6*B* and Table 3. None of the vehicle-treated control animals had tumor regression, 17% of the ATRA-treated mice had some tumor regression, 67% of the TPA-treated mice had some tumor regression, and 83% of the mice treated with TPA and ATRA had some tumor regression (Table 3, group B).

Table 3 *Effects of i.p. injections of TPA,[a] ATRA, or TPA+ATRA on the growth or regression of LNCaP prostate tumors in NCr mice: summary of data with individual mice*

Male NCr immunodeficient mice with LNCaP tumors received i.p. injection once a day with vehicle, TPA (0.16 nmol/g), ATRA (0.5 nmol/g), or TPA (0.16 nmol/g) + ATRA (0.5 nmol/g) in vehicle as described in "Materials and Methods" and Fig. 5. Mice in group A (13 mice/group) were treated for 21 days, and a subset of these mice (6 mice/group) were treated for an additional 25 days (group B) as described in Fig. 6. The effects of the various treatments on tumor growth or regression in individual mice are indicated.

Group	Treatment	No. of mice	Duration of treatment (days)	Percentage of animals with tumor growth	Percentage of animals with tumor regression
A	Vehicle control	13	21	100	0
	ATRA	13	21	69	31
	TPA	13	21	38	62
	TPA+ATRA	13	21	0	100
B	Vehicle control	6	46	100	0
	ATRA	6	46	83	17
	TPA	6	46	33	67
	TPA+ATRA	6	46	17	83

[a] TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ATRA, all-*trans*-retinoic acid.

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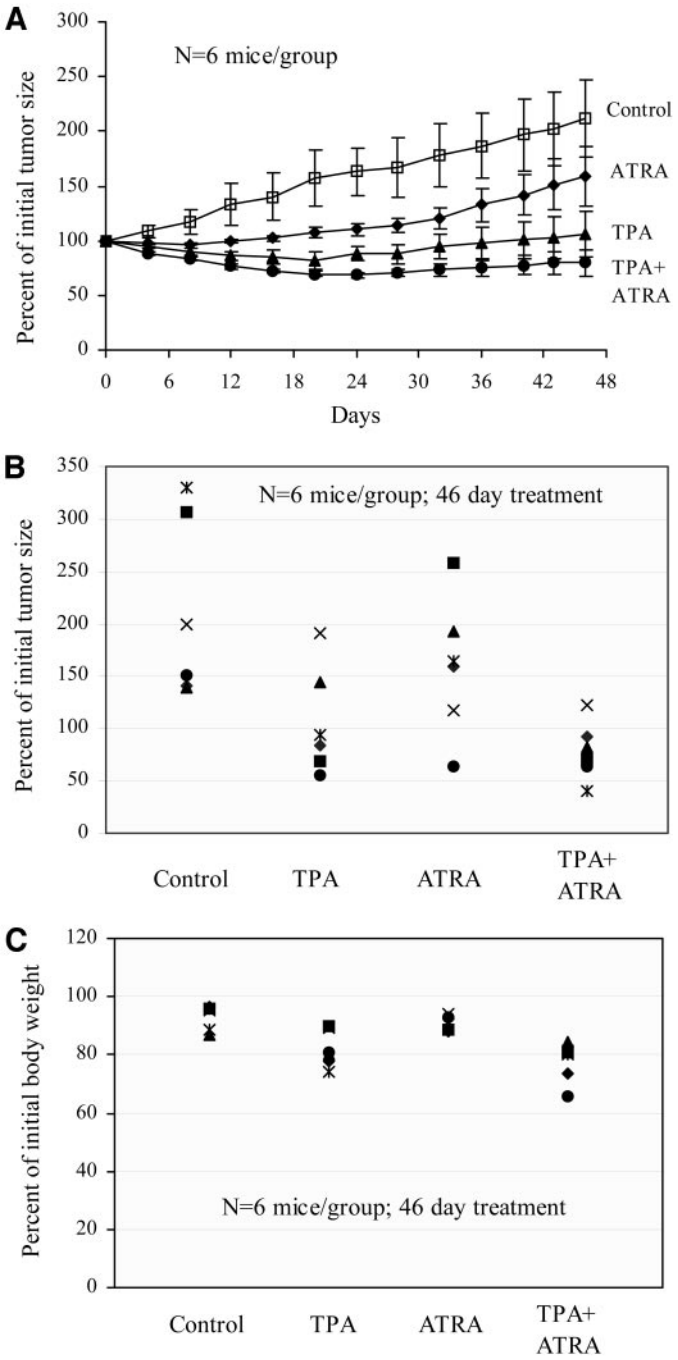

Fig. 6. Effects of i.p. injections of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or all-*trans*-retinoic acid (ATRA) alone or in combination for 46 days on the growth of LNCaP tumors and body weight of NCr immunodeficient mice. A subset of 6 mice/group from the animals described in Fig. 5 were continued on the same treatment as described in Fig. 5 for an additional 25 days (total of 46 days). Tumor size (length × width) was measured and expressed as a percentage of initial size. *A*, growth curve of LNCaP tumors in each group. Each value represents the mean ± SE. *B*, change in individual tumor size after treatment for 46 days. The mean ± SE for the percentage of initial tumor size for the mice was 211.1 ± 35.2 for the control group, 106.1 ± 21.0 for the TPA group, 159.2 ± 27.0 for the ATRA group, and 78.2 ± 11.3 for the TPA and ATRA group. *C*, change in individual body weight of mice after treatment for 46 days. The mean ± SE for percentage of initial body weight was 93.1 ± 1.8% for the control group, 83.7 ± 2.2% for the TPA group, 90.3 ± 1.1% for the ATRA group, and 77.4 ± 3.1 for the TPA and ATRA group.

The effects of the various treatments on body weight are described in Fig. 6*C*. The mean ± SE for the percentage of initial body weight (6 mice/group) after 46 days of treatment was 93.1 ± 1.8% for the control group, 83.7 ± 2.2% for the TPA group, 90.3 ± 1.1% for the ATRA group, and 77.4 ± 3.1% for the TPA+ATRA group. Statistical analysis with the Dunnett multiple comparison test showed that the differences in the percentage of initial body weight between the control group and the TPA group or between the control group and the TPA+ATRA group were statistically significant ($P < 0.05$). A comparison of data relating changes in body weight to changes in tumor size in individual mice from all of the 24 mice treated for 46 days indicated a relationship between these two parameters ($r = 0.531$) that is statistically different from zero ($P = 0.0075$). There was no statistically significant relationship between these two parameters in the 12 mice treated with TPA alone or in combination with ATRA for 46 days ($r = 0.03$).

The overall conclusions from these studies on the effects of treatment of tumor-bearing mice with TPA alone or in combination with ATRA for 21 or 46 days indicate strong inhibitory effects on tumor growth and enhanced tumor regression. Although inhibitory effects of TPA or TPA+ATRA on body weight were observed during our studies, particularly in mice treated with TPA alone or in combination with ATRA for 46 days, the effects of TPA and TPA+ATRA on body weight do not appear to be closely related to the effects of TPA or TPA+ATRA on tumor growth and regression, particularly during the first 21 days of treatment.

Effects of i.p. Injections of TPA or ATRA Alone or in Combination Once a Day for 46 Days on Proliferation and Apoptosis in LNCaP Tumors in Immunodeficient Mice

Male NCr mice with LNCaP tumors were treated with vehicle, TPA, ATRA, or TPA+ATRA for 46 days as described in Fig. 6*A*, and the animals were sacrificed 24 h after the last injection. Tumor size (length × width; cm^2) was measured in each mouse before sacrifice, and the weight of each tumor (g) was determined after sacrifice. An excellent relationship between tumor size (as measured in live animals) and tumor weight in individual mice was obtained ($r = 0.932$; Fig. 7), and the 95% confidence interval was 0.844–0.971. Because the extent of mitoses in tumors is a potential index of proliferation, and the extent of caspase 3 (active form)-positive cells in tumors is a potential index of cell death, we compared the effects of the various treatments on tumor growth and the ratio of the percentage of mitotic

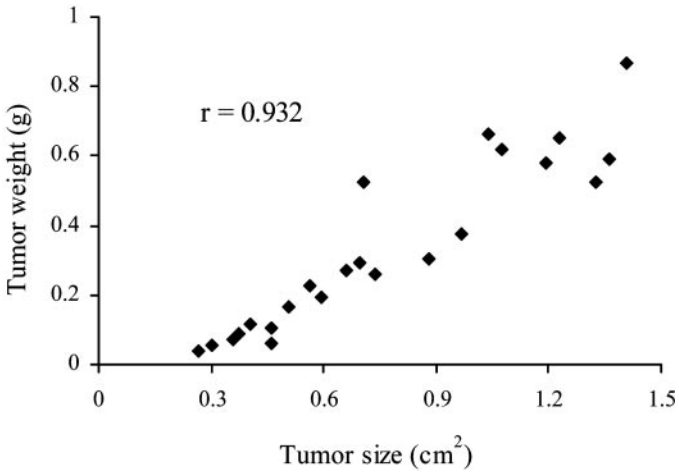

Fig. 7. Relationship between tumor size measured *in vivo* and tumor weight measured after sacrifice of animals. Male NCr mice with LNCaP tumors were treated with vehicle, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), all-*trans*-retinoic acid (ATRA), or TPA+ATRA for 46 days as described in Fig. 6. Tumor size (length × width, cm^2) was measured before sacrifice, and the weight of each tumor (g) was determined after sacrifice. The relationship between tumor size (as measured in live animals) and tumor weight at autopsy was determined and was highly correlated ($r = 0.932$) with a 95% confidence interval of 0.844–0.971.

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Table 4 *Effects of i.p. injections of TPA,[a] ATRA, or TPA+ATRA on the percentage of mitotic cells and the percentage of caspase 3 (active form)-positive cells in LNCaP tumors*

Male NCr mice with LNCaP tumors received i.p. injection once a day with vehicle or TPA, ATRA, or TPA in combination with ATRA in vehicle for 46 days as described in Fig. 6. The animals were sacrificed 24 h after the last injection. Mitotic cells were determined with a light microscope in H&E-stained tissue sections. Caspase 3 (active form)-positive cells were determined immunohistochemically as described in "Materials and Methods." Each value represents the mean ± SE.

Treatment	No. of animals	Percentage of mitotic cells	Percentage of caspase 3-positive cells	Ratio of percentage of mitotic cells/caspase 3-positive cells
Control	5	0.58 ± 0.06	0.31 ± 0.03	1.98 ± 0.31
TPA	6	0.50 ± 0.05	0.45 ± 0.03[b]	1.13 ± 0.11[b]
ATRA	6	0.54 ± 0.04	0.35 ± 0.03	1.62 ± 0.19
TPA+ATRA	6	0.39 ± 0.02[b]	0.47 ± 0.04[c]	0.87 ± 0.11[c]

[a] TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ATRA, all-*trans*-retinoic acid.
[b] $P < 0.05$.
[c] $P < 0.01$.

cells/percentage of caspase 3 (active form)-positive cells. We also determined whether this ratio was correlated with tumor growth in individual mice. The percentage of mitotic cells was decreased significantly in tumors from mice treated with TPA+ATRA but not in the other groups (Table 4), and similar results were observed for the BrdUrd labeling index (data not shown). Apoptosis as measured by the percentage of caspase 3 (active form)-positive cells in tumors was increased significantly in the TPA group and the TPA+ATRA group (Table 4), and the ratio of percentage of mitotic cells/percentage of caspase 3 (active form)-positive cells was decreased in these groups (Table 4). The ratio of the percentage of mitotic cells/percentage of caspase 3 (active form)-positive cells ± SE in tumors was 1.98 ± 0.31 for the vehicle-treated control group, 1.13 ± 0.11 for the TPA group, 1.62 ± 0.19 for the ATRA group, and 0.87 ± 0.11 for the TPA+ATRA group (Table 4). There was an excellent relationship in individual mice treated for 46 days between the percentage of initial tumor size *versus* the ratio of percentage of mitotic cells/percentage of caspase 3 (active form)-positive cells for the 23 tumors examined (Fig. 8). The *r* value for this relationship was 0.853, with a 95% confidence interval of 0.679–0.936.

DISCUSSION

In the present study, we demonstrated for the first time a synergistic inhibitory effect of TPA and ATRA on the growth of cultured LNCaP prostate cancer cells. Although treatment of these cells with a low concentration of TPA or ATRA alone had little or no effect on apoptosis, treatment of the cells with a combination of these agents resulted in a strong apoptotic effect (Table 1). In additional studies, we also observed a greater-than-additive effect of TPA and ATRA on the growth of the androgen-independent C4-2 cell line (see "Results"). The concentrations used to obtain synergistic effects (0.16–0.32 nM for TPA and 1 μM for ATRA) are clinically achievable levels (27, 32). The peak blood concentration of TPA ± SD in several patients who received an i.v. infusion of TPA (0.125 mg/m^2) was 2.84 ± 0.89 nM and ranged between 0.5 and 8.4 nM (27). The peak blood level of ATRA ± SD after treatment of patients with ATRA (15 mg/m^2) was 1.13 ± 0.68 μM (32). In the present study, daily i.p. injections of ATRA or TPA for 21 days inhibited the growth of well-established LNCaP tumors in immunodeficient mice, and tumor regressions were observed in several animals (Fig. 5; Table 3). Combined administration of TPA and ATRA resulted in some tumor regression in all of the animals treated for 21 days ($n = 13$) and in 83% of the animals treated for 46 days ($n = 6$; Table 3). Determination of blood levels of TPA in TPA-treated mice indicated clinically achievable blood levels and a half-life of 3–5 h (Fig. 4) that can serve as a guide for selecting dosing regimens for future studies on the *in vivo* effects of TPA on the growth of tumor xenografts in mice. Our results indicate that administration of lower dose levels of TPA than used in the present study but administered two or three times a day would achieve more sustained blood levels and might be more effective than the once-a-day dosing regimen of TPA used in the present study. Alternatively, administration of a higher dose of TPA one to three times a week may be more effective than the daily dosing regimen. Although more research is needed to determine an optimal dosing regimen, the results of the present study provide the first demonstration of an *in vivo* inhibitory effect of TPA on the growth of tumors in an animal model.

The inhibitory effect of ATRA treatment (0.5 nmol/g/day) on the growth of LNCaP tumors in immunodeficient mice contrasts with the lack of an inhibitory effect of ATRA (1–10 μM) on the growth of cultured LNCaP cells. Although we did not measure ATRA blood levels in the *in vivo* study, assuming equal distribution of ATRA throughout the mouse and the lack of metabolism, the maximum achievable concentration of ATRA would be 0.5 μM, and it is likely that the concentration of ATRA would be considerably less. The reason(s) why ATRA was inactive in the cell culture studies but active *in vivo* is not known but could be caused by the metabolism of ATRA to active metabolites *in vivo* or the combined effect of ATRA and endogenous constituents [possibly tumor necrosis factor (TNF)-α] that, in combination with ATRA, exert an inhibitory effect on tumor growth. Although the results of our studies indicate direct effects of TPA or TPA+ATRA on cultured LNCaP and C4-2 prostate cancer cells, the possibility that TPA and ATRA inhibit the growth of LNCaP tumors *in vivo* via an effect on androgen or prostate-specific antigen levels has not been excluded and requires further investigation.

Mechanistic studies indicated that administration of TPA alone or in combination with ATRA once a day for 46 days to tumor-bearing NCr mice enhanced apoptosis in the tumors as measured by increased numbers of caspase 3 (active form)-positive cells, and an inhibitory effect of administration of TPA+ATRA on mitosis in the tumors was also observed. These effects were seen at 24 h after the last dose of TPA or TPA+ATRA, and it is possible that greater effects would have been observed if the animals had been sacrificed at shorter time intervals after the last dose of TPA or TPA+ATRA. It was of

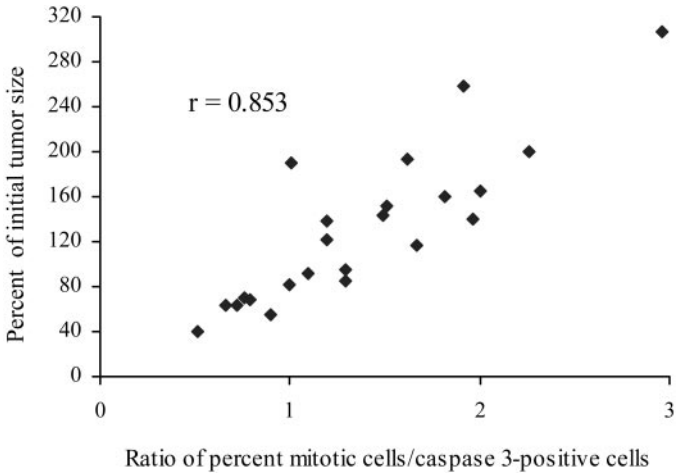

Fig. 8. Relationship between tumor growth and the ratio of percentage of mitotic cells/caspase 3 (active form)-positive cells in individual mice. The percentage of initial tumor size after 46 days of treatment with vehicle, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), all-*trans*-retinoic acid (ATRA), or TPA+ATRA was determined for the 23 mice described in Table 4, and the ratio of percentage of mitotic cells/caspase 3 (active form)-positive cells was also determined in each tumor. The *r* value for the relationship between percentage of initial tumor size *versus* the ratio of percentage of mitotic cells/caspase 3 (active form)-positive cells for the 23 tumors was 0.853 with a 95% confidence interval of 0.679–0.936.

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considerable interest that the ratio of the percentage of mitotic cells to the percentage of apoptotic cells in the tumors from individual mice at the end of the study was closely associated with tumor growth during the 46-day treatment regimen ($r = 0.853$; Fig. 8). In another study, Garzotto *et al.* (23) showed that γ-irradiation (^{137}Cs) or an i.v. injection of TPA into the retro-orbital plexus (80 ng/g; 0.13 nmol/g body weight) had a minimal effect on apoptosis in orthotopically transplanted LNCaP tumors in immunodeficient mice, but treatment of the mice with a combination of γ-radiation and TPA resulted in apoptosis in the tumors at 24 h after the treatment. This report also indicated that TPA administration had a protective effect on γ-radiation-induced damage to the colon (23). The results of the present study and the study by Garzotto *et al.* (23) provide a rationale for clinical trials on the effects of TPA alone or in combination with ATRA and/or γ-radiation in patients with prostate cancer.

Vitamin A and its natural and synthetic analogs (retinoids) induce apoptosis in prostate cancer cells *in vitro* and in animal models (33). ATRA has been shown to inhibit the growth and induce apoptosis in normal and malignant epithelial prostate cells (24, 25), and it strongly enhances the apoptotic effect of docetaxel in Du-145 and LNCaP prostate cancer cells (34). Although it is known that the effects of retinoids are mainly mediated by two classes of nuclear receptors, the retinoic acid receptors and retinoid X receptors (33), the molecular mechanisms by which retinoids induce apoptosis in prostate cancer cells remain largely unknown. Induction of apoptosis in prostate cancer cells by several retinoids appears to be associated with down-regulation of Bcl-2 expression (33, 35), induction of insulin-like growth factor-binding protein 3 (36), and induction of tissue transglutaminase (24), an enzyme that accumulates in certain cells undergoing apoptosis. Retinoid X receptor α was found to interact with insulin-like growth factor-binding protein 3, and insulin-like growth factor-binding protein 3-induced apoptosis was abolished in retinoid X receptor α knockout cells. The combination of ATRA and the organic arsenical melarsoprol synergistically induced apoptosis in Du-145 and PC-3 cells *in vitro* and in immunodeficient mice (37). Although ATRA was shown to enhance TPA-induced differentiation of myeloid leukemia cells (14, 38) and to enhance TPA-induced growth inhibition of breast and lung cancer cells (39), the effects of combinations of TPA and ATRA at clinically relevant concentrations on the growth and death of human prostate cancer cells were not reported previously. As indicated above, the present study demonstrates a synergistic inhibitory effect of TPA and ATRA on the growth of LNCaP prostate cancer cells.

The mechanism of TPA-induced apoptosis in prostate cancer cells remains unclear. TPA-induced apoptosis in LNCaP cells was accompanied by prolonged translocation of protein kinase C (PKC) α to nonnuclear membranes, implying prolonged activation, and TPA-resistant LNCaP cells had down-regulation of PKCα (20). Unpublished observations[7] from our laboratory showed that a low concentration of 7-hydroxystaurosporine (UCN-01; 50 nM) abrogated TPA-induced growth inhibition and apoptosis in LNCaP cells. Because a low concentration of 7-hydroxystaurosporine has a selective inhibitory effect on PKCα and PKCβ (IC_{50} = 29–34 nM; Ref. 40), and PKCβ is not detected in LNCaP cells (20), these findings support the possibility that TPA-induced growth inhibition and TPA-induced apoptosis in LNCaP cells may be mediated in part through PKCα. Other studies provided evidence that TPA-induced apoptosis in LNCaP cells is mediated via PKCδ and that overexpression of this isoform of PKC resulted in increased sensitivity of LNCaP cells to TPA-induced apoptosis (21). Furthermore, TPA-induced apoptosis in LNCaP cells is associated with translocation of a nuclear hormone orphan receptor, TR3/Nur77, from the nucleus to the mitochondria with induction of cytochrome *c* release, thus providing evidence for another potential mechanism for TPA-induced apoptosis (41). TPA-induced apoptosis in LNCaP cells is also associated with increased ceramide synthase activity and increased levels of cellular ceramide (15), which provides an additional potential mechanism for TPA-induced apoptosis.

Although the results of our present study and studies by other investigators indicate that LNCaP cells are highly sensitive to TPA-induced growth inhibition and increased apoptosis, PC-3 and Du-145 prostate cancer cells are considerably less sensitive (42). Furthermore, long-term treatment of LNCaP cells with TPA resulted in a TPA-resistant phenotype (20). One approach for enhancing the effectiveness of TPA and overcoming the resistance of prostate cancer cells to TPA-induced apoptosis is to use combinations of TPA with other anticancer agents. The present study demonstrates that a combination of TPA and ATRA at clinically achievable concentrations synergistically inhibits growth and increases apoptosis in LNCaP cells *in vitro*. Moreover, treatment of NCr mice bearing LNCaP tumors with a combination of TPA and ATRA results in tumor regression, whereas administration of TPA or ATRA alone at the same dose used in the combination predominantly suppresses tumor growth. Although treatment of tumor-bearing mice with TPA, ATRA, or TPA+ATRA had strong inhibitory effects on tumor growth for the first 21 days of the study, the therapeutic effects of these treatments decreased after 21 days (Fig. 6). Whether modification of the dosing regimen or giving TPA in combination with additional anticancer agents or γ-irradiation would provide more effective therapy requires further investigation.

The molecular mechanisms by which TPA and ATRA synergistically inhibit growth and induce apoptosis in LNCaP cells are not known. The results of our studies indicated a TPA-dependent increase in TNF-α in LNCaP cells,[7] which is in agreement with an earlier study (43). Additional studies showed an effect of TNF-α to inhibit the growth of LNCaP cells and indicated that treatment of LNCaP cells with a combination of TNF-α and ATRA caused a greater-than-additive inhibitory effect on the growth of these cells.[7] Recent studies have shown that TNF-α synergized with ATRA to induce differentiation in myeloid leukemia cells (44) and apoptosis in glioblastoma cells (45). Our results are consistent with a mechanistic explanation for the synergistic effect of TPA+ATRA on LNCaP cell growth via TPA-induced formation of TNF-α that synergizes with ATRA to inhibit the growth of LNCaP cells. The effect of this combination on apoptosis has not yet been investigated. ATRA has been shown to increase the expression of PKCα in breast cancer cells (46) and to increase the expression of PKCβ in myeloid leukemia cells (47). A combination of TPA and ATRA is known to synergistically activate PKC (48), and ATRA is also known to increase the concentration of cellular ceramide (49). In unpublished observations,[7] we found that a low concentration of 7-hydroxystaurosporine (UCN-01; 50 nM) abrogated the synergistic inhibitory effect of TPA in combination with ATRA on the growth of LNCaP cells. This result suggests that PKCα may play a role in the synergy produced by TPA and ATRA.

There are only a few reports on systemic effects of TPA in animals. Daily i.p. injections of TPA (0.16 nmol/g body weight) for 46 days in the present study caused some loss in body weight (Fig. 6), but gross autopsy of these animals failed to reveal abnormalities. Garzotto *et al.* (23) injected TPA (100 ng/g; 0.16 nmol/g body weight) i.v. via the retro-orbital plexus once a week for 4 weeks, and detailed autopsy examination of the animals 6 months later failed to find abnormalities. An i.p. injection or oral intubation with 10 μg of TPA in transgenic mice expressing the Activator Protein-1 (AP-1) luciferase reporter gene markedly stimulated AP-1 activity in the skin and esophagus, and mitogen-activated protein kinase phosphorylation was also in-

[7] X. Zheng, X-X. Cui, G. E. Avila, S. Lee, and A. H. Conney, unpublished observations.

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creased (50). The i.v. administration of a high dose of TPA to rabbits or sheep has been reported to induce respiratory distress and cause an inflammatory response in the lung (51–53). Whether a similar effect can occur with the low doses of TPA used in cancer therapy studies in animals and humans is not known. Common side effects associated with i.v. infusions of 0.25–1 mg of TPA in humans were vein irritation and transient fever. Transient dyspnea also occurred in an occasional individual (11–13), and this effect may be related to TPA-induced lung inflammation observed in animals. The results of the clinical trials indicate an acceptable toxicity profile at the dose levels tested.

In summary, the present report indicates an inhibitory effect of daily i.p. injections of TPA or ATRA on the growth of LNCaP prostate tumors in immunodeficient mice, and tumor regressions were also observed. Substantially greater effects on tumor growth and tumor regression were observed when TPA was administered in combination with ATRA. Administration of TPA alone or in combination with ATRA increased apoptosis in the tumors. Because the dose of TPA used in the present study resulted in clinically achievable blood levels, clinical trials with TPA alone or together with ATRA in patients with prostate cancer may be warranted.

ACKNOWLEDGMENTS

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Inhibitory Effect of 12-*O*-Tetradecanoylphorbol-13-acetate Alone or in Combination with All-*trans*-Retinoic Acid on the Growth of LNCaP Prostate Tumors in Immunodeficient Mice

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