Antitumor Synergy of CV787, a Prostate Cancer-specific Adenovirus, and Paclitaxel and Docetaxel

De-Chao Yu, Yu Chen, Jeanette Dilley, Yuanhao Li, Millicent Embry, Hong Zhang, Natalie Nguyen, Pinky Amin, Joe Oh, and Daniel R. Henderson

Calydon, Incorporated, Sunnyvale, California 94089

Abstract

CV787, a PSA+ prostate cell-specific adenovirus variant, is currently in Phase I/II clinical trials for the treatment of prostate cancer. We have previously demonstrated that a single administration of CV787 at 1 x 10^11 particle/animal could eliminate established tumors within 6 weeks in nude mouse xenografts (Yu et al., Cancer Res., 59: 4200–4203, 1999). We now demonstrate that CV787-mediated replication-dependent cytotoxicity is synergistic with the chemotherapeutic agents paclitaxel (Taxol) or docetaxel (Taxotere) both in vitro and in vivo. In vitro, cells were pretreated with CV787 24 h before taxane, pretreated with taxane 24 h before CV787, or treated with both agents simultaneously. Cell viability was determined at various time points by 3-[4,5-dimethylthiazole-2–4]-2,5-diphenyl-2H-tetrazolium bromide assay, and virus yield was examined by plaque assay. Addition of taxane to CV787 resulted in a synergistic increase of cytotoxicity toward the human prostate cancer cell line LNCaP, regardless of the timing of administration. There was no reduction in virus replication or specificity of CV787-based cytopathogenicity for prostate cancer cells (approximately 10,000 to 1) with the taxanes. p53 expression was significantly elevated in the cells treated with CV787 and taxane. In vivo, using the PSA+ LNCaP xenograft model of prostate cancer, a single i.v. dose of 1 x 10^6 particles CV787 and docetaxel in combination eliminates large preexistent distant tumors. Toxicity studies do not show a synergistic increase of toxicity of CV787 and taxane. These experiments demonstrate a synergistic antitumor efficacy for CV787 when combined with taxane and demonstrate an in vivo single-dose curative therapeutic index for CV787 of over 1000:1.

Introduction

Prostate cancer is the most commonly diagnosed neoplasm in men, accounting for 29% of all of new cancers. In 2000, an estimated 180,400 men will be diagnosed with prostate cancer and approximately 32,000 will die (1). Surgery and radiation therapy (external beam or brachytherapy seed implantation) remain the most common therapeutic modality for the treatment of clinically localized prostate cancer (2). Once prostate cancer enters a metastatic stage, the current treatment is androgen ablation therapy, which provides relief from otherwise uncontrollable bone pain and increases life expectancy by 6–18 months in 70% of men (3). For many years, it was felt that chemotherapy did not play any role in the treatment of advanced prostate cancer. However, this negative impression may be starting to change because activity is being seen with new drugs and drug combinations (4) and in particular with the use of docetaxel (5, 6).

We previously developed an adenovirus variant, CV787, that replicates preferentially in prostate cells that produce PSA. This was accomplished by engineering two prostate-specific transcription response elements into the adenovirus genome to control the expression of essential viral genes (7, 8). Animal studies have demonstrated that a single i.v. administration of CV787 at a dose of 1 x 10^11 particles/animal can eliminate distant prostate tumor xenografts in Balb/C nu/nu mice (7). This virus has entered Phase I/II clinical trials for the treatment of locally recurrent prostate cancer by intraprostatic injection and of metastatic disease by i.v. administration.

Our animal studies have also indicated that the lethal dose of CV787 (LD100) for Balb/C nu/nu mice is 3 x 10^11 particles. Because the curative dose of CV787 administered i.v. as a single bolus is 1 x 10^11 particles, the curative therapeutic window is narrow. Although conventional cancer therapies (surgery, chemotherapy, and radiation) are often effective at curing early-stage disease, few human metastatic cancers are curable with a single modality. In an attempt to widen the single-dose curative therapeutic window of CV787, we explored a neoadjuvant therapy that consisted of combining CV787 with chemotherapeutic agents.

In the present study, we examined the efficacy of CV787 in combination with taxane (paclitaxel or docetaxel) against human prostate cancer cells using single doses. Synergistic efficacy was observed both in vitro and in vivo. Our in vivo studies show that the combination of CV787 with docetaxel at human therapeutic doses can eliminate LNCaP xenografts within 3–4 weeks using 1 x 10^8 particles of CV787. This result opens a single-dose curative therapeutic window for CV787 of over 1000:1 in this prostate cancer model.

Materials and Methods

Cell Lines and Culture. The human LNCaP (prostate carcinoma), HBL-100 (breast epithelia), OVCAR-3 (ovarian carcinoma), and HepG2 (liver hepatocellular carcinoma) cell lines were obtained from the American Type Culture Collection (Rockville, MD) and cultured as described (7).

Chemotherapeutic Agents and Virus. Paclitaxel (Taxol; Bristol-Myers Squibb, Princeton, NJ) and docetaxel (Taxotere; Rhone-Poulenc Rorer Pharmaceuticals, Inc., Collegeville, PA) were purchased from the Stanford Pharmacy (Stanford Medical Center, Palo Alto, CA). These agents were diluted with medium without fetal bovine serum just before use for in vitro studies and with 0.9% NaCl for in vivo studies.

CV787 is a prostate-specific replication competent adenovirus variant with the rat probasin promoter driving the Ad5 E1A gene and with the human prostate-specific antigen promoter and enhancer driving the Ad5 E1B gene (7).

Cell Viability. MTT assays were performed by seeding LNCaP, HBL-100, OVCAR-3, HepG2, and 293 cells at 5000 cells/well in a 96-well plate (Falcon) 48 h before infection as described previously (9), with modifications. Cells were either infected with CV787 at an MOI of 2 PFU/cell or treated with the indicated chemotherapeutic agents (paclitaxel at 6.25 nm and docetaxel at 3.12 nm). Cell viability was measured at the times indicated by removing the media
and replacing it with 50 μl of a 1 mg/ml solution of MTT (Sigma Chemical Co., St. Louis, MO) and incubating for 3 h at 37°C. After removing the MTT solution, the crystals that remained in the wells were solubilized by the addition of 50 μl of isopropanol followed by vigorous shaking. The absorbance was determined using a microplate reader (Molecular Dynamics) at 560 nm (test wavelength) and 690 nm (reference wavelength). The percentage of surviving cells was estimated by dividing the A_560 nm − A_690 nm of virus-infected cells by the A_560 nm− A_690 nm of mock infected cells. Twelve replicate samples were taken for each time point, and each experiment was repeated at least three times.

Statistical Analysis. The dose-response interactions between taxane and CV787 at the point of IC_{50} were evaluated by the isobologram method of Steel and Peckham (10) as modified by Aoe et al. (11). The IC_{50} was defined as the concentration of drug that produced 50% cell growth inhibition, i.e., 50% reduction in absorbance. Cells were exposed to drugs sequentially for 24 h, and cell viability was determined by the MTT assay after 6 days. The dose-response curves were plotted with CurveExpert (Version 1.34) on a semilog scale as a percentage of the control, the absorbance of which was obtained from the samples not exposed to the drugs. IC_{50} value of CV787 and taxane in LNCaP was then determined. On the basis of the dose-response curves of CV787 alone and taxane alone, isobolograms (three isoeffect curves, mode 1 and mode 2 lines) were computed. The envelope of additivity, surrounded by mode 1 and mode 2 isobologram lines, was constructed from the dose-response curves of CV787 alone and taxane alone. The observed data were compared with the predicted maximum and minimum data for the presence of synergism, additivity, or antagonism by a statistical analysis using the Stat View 4.01 software program (Abacus Concepts, Berkeley, California). When the data points of the drug combination fall within the area surrounded by mode 1 and/or mode 2 lines (i.e., within the envelope of additivity), the combination is described as additive. A combination that gives data points to the left of the envelope of additivity can be described as supraadditive (synergism), and a combination that gives data points to the right of the envelope of additivity can be described as subadditive (antagonistic; Ref. 12). Fractional tumor volume relative to untreated controls was determined as described previously (13).

One-step Growth Curve and Virus Yield. One-step growth curves of CV787 in the presence or absence of docetaxel were performed in LNCaP and 293 cells as described (8).

Immunohistology. LNCaP cells treated with CV787, taxane, or both CV787 and taxane were incubated for the indicated times. Cells were washed with cold PBS and lysed for 30 min on ice in 50 mM Tris (pH 8.0), 150 mM NaCl, 1% IGEPAL CA360 [(octyphenoxy)polyethoxyethanol] (NP40 equivalent from Sigma), 0.5% sodium deoxycholate, and protease inhibitor cocktail (Roche, Palo Alto, California). After 30-min centrifugation at 4°C, the supernatant was removed and protein concentration was determined by the ESI protein assay kit (Roche). Fifty μg of protein/lane were separated on 8–16% SDS-PAGE and electroblotted onto Hybond enhanced chemiluminescence membranes (Amersham Pharmacia, Buckinghamshire, England). The membranes were blocked overnight in PBS with 0.1% Tween 20 supplemented with 5% nonfat dry milk. Primary antibody incubation was done at room temperature for 2–3 h with PBS with 0.1% Tween 20% nonfat dry milk-diluted antibody, followed by wash and 1-h incubation with diluted horseradish peroxidase-conjugated secondary antibody. Enhanced chemiluminescence (Amersham Pharmacia) was used for detection. Antibodies for p53 and poly(ADP-ribose) polymerase were from Roche. Antibodies against Fas/Fas-L, caspase 7, Bcl-2, Bcl-XL, Bax, and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California). All of the antibodies were used according to manufacturer’s instructions. For quantifying the bands, the gels were scanned and bands were analyzed by Multi-Analyst software (Bio-Rad).

In Vivo Antitumor Efficacy. LNCaP xenografts were established as described (7, 14). Mice that bore tumors were randomized into groups of four. The first group received 1 × 10^5 particles of CV787 at day 1 via the tail vein i.v. CV787 was diluted in 0.1 ml lipothilized buffer (5% sucrose, 1% glycine, 1 mM MgCl_2, 0.05% Tween 80 in 10 mM Tris buffer) and injected into the tail vein using a 28-gauge needle. The second group was given taxane only. Paclitaxel was i.p. administered at a dose of 20 mg/kg, daily for 4 days starting at day 2. Docetaxel was i.v. administered at a dose of 5 or 12.5 mg/kg at day 2, 5, and 8. The third group was given CV787 (i.v.) at day 1 and taxane at the same doses and schedule as the second group. As a control, a fourth group was treated with 0.1 ml of normal saline (i.e., control) i.v. at day 1 and then i.p. or i.v. for 4 days. The dose and route of administration of paclitaxel were selected according to studies in nude mice (15, 16). For docetaxel, the dose was selected based on the human clinical dose (Rhone-Poulenc Rorer Pharmaceuticals, Inc.) and determined by a dose-range finding study in nude mice (see “Results”). Tumor volume and PSA levels were measured as described previously (7, 14). Federal and institutional guidelines for animal care were followed.

Immunohistochemistry. Four groups of mice (n = 6) were treated with vehicle, CV787 (1 × 10^10 particles/animal), paclitaxel (15 mg/kg), or a combination of CV787 and paclitaxel. Half of the animals were sacrificed on day 9 and the other half on day 16. Histology methods for adenovirus were as described (7).

Apoptotic cells were detected using M30 monoclonal antibody with reagents from the M30 CytoDEATH kit (Roche Molecular Biochemicals, Indianapolis, IN) as suggested by the manufacturer. Paraffin-embedded tumor sections were heated in citric acid buffer for 15 min to retrieve antigen, hybridized with M30 antibody, then counterstained with Harries hematoxylin (Roche Molecular Biochemicals). The stained sections were analyzed under a light microscope, and pictures of representative sections were taken.

Results

Toxicity of CV787 in Balb/C nu/nu Mice. Our Balb/C nu/nu animal studies showed that the lethal dose of CV787 (LD_0 and LD_100) administered as a single bolus i.v. is 1 × 10^{11} and 3 × 10^{11} particles, respectively. Interestingly, the LD_100 requires slightly more virus particles for CV787 (3 × 10^{11} particles), a virus containing the E3 region, than for CN706 (2.5 × 10^{11} particles), a virus not containing the E3 region (Ref. 14; and data not shown). However, as shown previously, the range between the LD_0 and LD_100 is surprisingly narrow, a difference of only 3-fold. Balb/C nu/nu animals die 4–6 days after i.v. injection of a LD_100 dose of CV787 from liver toxicity (14).

Combination of CV787 with Paclitaxel or Docetaxel Is Synergistic in LNCaP Cells in Vitro. To study the potential interaction between CV787 and chemotherapy in vitro, the effectiveness of the combined treatment of several concentrations of paclitaxel or docetaxel with CV787 at various MOI was evaluated in the PSA-producing prostate carcinoma LNCaP cell line. Cells were treated with CV787 and paclitaxel or docetaxel, and the cell viability was determined at various time points by the MTT assay. Several concentrations of docetaxel and paclitaxel were tested before the optimal concentrations were determined for further study, 6.25 nM for paclitaxel and 3.12 nM for docetaxel. Taxane concentrations were chosen based on the dose-response curves for each cell line, such that the selected drug concentrations show greater combined efficacy with CV787 but minimal cell killing when treated with the same dose of taxane alone. For example, infecting LNCaP cells with CV787 at an MOI of 0.01 resulted in 80% cell survival 8 days after infection, whereas paclitaxel at a dosage of 6.25 nm resulted in 80% survival 8 days after treatment. However, when CV787 and paclitaxel were combined at these concentrations, cell survival dropped to 12% 8 days after treatment (Fig. 1A).

Isobolograms were generated from the models to determine the presence of synergy, additivity, or antagonism between CV787 and paclitaxel. Dose-response curve analysis indicated that the IC_{50} in LNCaP cells at day 5 for CV787 and paclitaxel was 0.17 MOI and 10.3 nM, respectively (data not shown). Fig. 1B shows isobologram representation of the statistical modeling used to analyze the drug interactions between CV787 and paclitaxel. The combined data points fell within the envelope of additivity or were smaller than that of the predicted minimum data, which indicates that sequential exposure to CV787 followed by paclitaxel produced synergistic effects.

Enhanced cytotoxicity was also observed in the combination treatment of CV787 and docetaxel. LNCaP cells were first incubated with CV787 at an MOI of 0.01 and 24 h later were treated with docetaxel.
at 3.12 nM. Cell viability was determined by MTT as shown in Fig. 1C. Combination treatment showed 10% cell survival at day 8 after treatment, whereas CV787 alone produced 80% cell survival and docetaxel alone showed 86% cell survival in prostate carcinoma LNCaP cells.

Isobolograms were also generated to show the synergy between CV787 and docetaxel. Dose-response curve analysis indicated that the IC50 at day 5 in LNCaP cells for CV787 and docetaxel was 0.368 MOI and 8.14 nM, respectively. Fig. 1D shows isobologram representation of the statistical modeling used to analyze the interaction between CV787 and docetaxel. The combined data points fell to the left of the envelope of additivity, or, restated, the IC50 in LNCaP cells of CV787 in combination with docetaxel occurred at smaller doses than that predicted from the use of CV787 or docetaxel alone. Thus, sequential exposure to CV787 followed by docetaxel produced synergistic effects.

Taxane Increases CV787 Burst Size in LNCaP Cells. Paclitaxel and docetaxel are antineoplastic agents belonging to the taxane family. They are novel antimicrotubule agents that promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cellular functions (17). In addition, the taxanes induce abnormal arrays or “bundles” of microtubules throughout the cell cycle and multiple asters of microtubules during mitosis (18, 19). One possible explanation for the synergy seen with taxane and CV787 is that taxane may augment the ability of CV787 to replicate in LNCaP cells.

To examine the effect of paclitaxel and docetaxel on virus replication, we performed the one-step growth curve. LNCaP cells were infected with CV787 at an MOI of 1 for 4 h, followed by incubation in RPMI 1640 that contained docetaxel at a final concentration of 3.12 nM. Cells were harvested at various times after infection, and the number of infectious virus particles was determined on 293 cells by standard plaque assay (7). As shown in Fig. 2, although the initial rate of increase of CV787 in cells treated with CV787 and docetaxel was similar to that of cells treated with CV787 alone, a plateau was reached for CV787 at approximately 72 h after infection and at approximately 96 h after infection for CV787 and docetaxel. Cells treated with CV787 and docetaxel produced 30,000 PFU/cell, whereas the cells infected with CV787 alone generated about 15,000 PFU/cell. Thus, docetaxel does not inhibit CV787 replication but actually increases virus replication efficiency. A similar result was obtained in a parallel study with paclitaxel (data not shown).

Taxane Does Not Alter the Specificity of CV787. To evaluate whether the addition of taxane changed the specificity of CV787 cytotoxic activity, we first tested the viability of various infected cell lines using the MTT assay to measure mitochondria activity. 293, LNCaP, HBL-100, and OVCAR-3 cells were infected with CV787 at an MOI of 0.1 in the presence or absence of paclitaxel at a concentration of 6.25 nM. The percentage of cell viability versus time after treatment was plotted in Fig. 3. The combination of CV787 and paclitaxel was toxic to 293 and LNCaP cells but not to HBL-100 and OVCAR-3 cells. There were few surviving LNCaP cells 9 days after infection.
virus yield was determined by plaque assay on 293 cells. Treatment with CV787 and paclitaxel or docetaxel produced a similar result. Results presented in Fig. 4 show that prostate cancer cells (LNCaP) were infected with either CV787 at an MOI of 0.1 or the same amount of CV787 plus taxane. The taxane concentration in the medium was equal for both paclitaxel (3.12 nM) and docetaxel (3.12 nM). Progeny virus yield was determined 72 h after infection by plaque assay on 293 cells. Cell viability was determined by MTT assay; error bars equal the SE.

6.25 nM for paclitaxel and 3.12 nM for docetaxel. Progeny virus yield was determined 72 h after infection by plaque assay on 293 cells. In contrast, the viability of HBL-100 and OVCAR-3 cells treated with CV787 and taxane also produced 5,000–10,000-fold lower virus, similar to the nonpermissive cells infected with CV787 alone. These experiments indicate that CV787 in the presence of taxane replicates efficiently in prostate cancer cells but retains its attenuated phenotype in nonprostate cancer cells.

Combination of Taxane and CV787 Increases the p53 Expression. To address the synergistic mechanism behind combination treatment, LNCaP cells were treated with various agents, and the expression of apoptotic-related protein markers was compared by Western blot. The treatments for LNCaP cells were grouped as (a) docetaxel alone at 6.0 nM; (b) CV787 alone at MOI 0.5; and (c) CV787 (MOI = 0.5) and docetaxel (6.0 nM) together. For each treatment group, cells were collected at different time points and subjected to various antibodies by Western blot. Under these experimental conditions in the first 48 h after treatment, the combination of CV787 and taxane increased p53 expression up to 2–8-fold compared with virus alone or drug alone at 24 or 48 h (Fig. 5).

In contrast, the apoptotic indicators caspase-7 and poly(ADP-ribose) polymerase did not show a significant change (data not shown). In addition, the combination of CV787 and taxane did not change Fas/Fas-L or Bcl-2, Bcl-XL, and Bax expression compared with the single agent group (data not shown). Previously, it was suggested that paclitaxel-induced apoptosis was not mediated by Bcl-2 family change (20, 21). In the current study, we did not observe a significant change of Bcl-2 expression in the cells treated with docetaxel alone, CV787 alone, or docetaxel and CV787. Liu and Stein (22) have reported that paclitaxel-treated LNCaP cells experienced alteration in bcl-XL and Bax expression. However, under our condition of low concentration of docetaxel, there was no dramatic change detected (data not shown). From the increased p53 expression, p53-dependent apoptosis may play a major role in the synergy of CV787 and taxane. However, further study is essential to understand this mechanism (23–25).

Synergistic Efficacy of Taxane with CV787 in Vivo. The in vivo antitumor efficacy of CV787 in combination with taxane was assessed in the LNCaP mouse xenograft model. We have shown previously that a single i.v. administration of CV787 at 1 × 10^11 particles/animal can eliminate s.c. xenograft tumors in 6 weeks (7). These data were extended using studies up through 10 weeks. Established human prostate tumors (LNCaP cells) were treated with vehicles, CV787 (1 × 10^10 particles/animal), paclitaxel (20 mg/kg), or both CV787 and paclitaxel. For the combination treatment, animals received i.v. injection of 3.12 nM paclitaxel and CV787 plus taxane was nearly 90% throughout the course of the experiment, similar to that of cells treated with paclitaxel alone.

To further assess the specificity of the combination treatment of CV787 and taxane, viral replication efficiency in two permissive cell lines, LNCaP and 293, and three nonpermissive cell lines, HepG2 (liver hepatocellular carcinoma), HBL-100 (breast epithelia), and OVCAR-3 (ovarian carcinoma), was examined. These five cell lines were infected with either CV787 at an MOI of 0.1 or the same amount of CV787 plus taxane. The taxane concentration in the medium was 6.25 nM for paclitaxel and 3.12 nM for docetaxel. Progeny virus yield was determined 72 h after infection by plaque assay on 293 cells. Results presented in Fig. 4 show that prostate cancer cells (LNCaP) treated with CV787 and paclitaxel or docetaxel produced a similar burst size to the cells infected with CV787 alone, which produced about 1000 PFU/cell, again suggesting that taxane does not inhibit CV787 replication. As expected, CV787 replicated poorly in the nonprostate cancer cells (HepG2, HBL-100, and OVCAR-3), producing 5,000–10,000-fold lower virus yield compared with the burst size in LNCaP cells. The same is true in the combination treatment group. Nonprostate cancer cells treated with CV787 and taxane also produced 5,000–10,000-fold lower virus, similar to the nonpermissive cells infected with CV787 alone. These experiments indicate that CV787 in the presence of taxane replicates efficiently in prostate cancer cells but retains its attenuated phenotype in nonprostate cancer cells.

Fig. 3. Effect of docetaxel on the replication-mediated cytotoxicity of CV787. 293, LNCaP, HBL-100, and OVCAR-3 cells were infected with CV787 (MOI = 0.01) 24 h before exposure to docetaxel (3.12 nM). Cell viability was determined by MTT assay; error bars equal the SE.

Fig. 4. Effect of taxane on the burst sizes of CV787 in PSA^- and PSA^+ cells. Cells were infected at a MOI of 0.1 and cultured in the medium with or without taxane (6.25 nM of paclitaxel or 3.12 nM of docetaxel). Cells were harvested 72 h after infection, and virus yield was determined by plaque assay on 293 cells.

Fig. 5. Immunoblot of p53 expression in LNCaP cells under different treatments. Cells were treated with (a) docetaxel alone; (b) CV787 alone; and (c) CV787 and docetaxel together. Cells were collected at 8, 24, and 48 h. Protein (50 μg) was loaded in each lane for SDS-PAGE followed by immunoblot with anti-p53 antibody and anti-actin antibody.
tions with either CV787 or vehicle, and 24 h later, paclitaxel was administered i.p. daily for 4 days. The tumor volume data presented in Fig. 6A show that there was a significant decrease in tumor volume between control and all of the treatment groups. In this study, single doses of CV787 or four doses of paclitaxel over 4 days were effective in producing partial tumor regression 7 weeks or 2 weeks after treatment, whereas the combination produced a near complete regression within 2 weeks (Fig. 6A). Four weeks after treatment, relative tumor volume decreased to 3% of baseline (from 418 mm$^3$ to 14 mm$^3$) for the combination treatment group and 162% of baseline for the CV787 group. These changes were statistically significant by Student’s $t$ test ($P < 0.05$) for the comparison of the combination treatment of CV787 with paclitaxel to any of the vehicles, CV787 or paclitaxel alone. Additionally, serum PSA levels in mice that received injections with vehicle increased, whereas the levels in mice that received injections with CV787 and paclitaxel decreased to $\sim$2% of their starting values within 4 weeks (data not shown).

Table 1 summarizes relative tumor volume of control and treated groups on four different time points. Combination therapy showed more than additive effect (e.g., synergy) on tumor growth inhibition. On day 21, there was 4.4-fold improvement in antitumor activity in the combination group when compared with the expected additive effect. At this time point, CV787 alone or paclitaxel alone inhibited tumor growth by 20% or 70%, respectively (fractional tumor volume, 0.815 mm$^3$ and 0.287 mm$^3$, respectively) when compared with the control group (Table 1). With time, there was a progressive improvement in antitumor activity. On day 42, the CV787 and paclitaxel combination group showed a 9.2-fold higher inhibition of tumor growth over additive effect (expected fractional tumor volume). These data demonstrated a synergistic efficacy between CV787 and paclitaxel in LNCaP xenografts.

A synergistic effect was also observed in the combination treatment of xenograft tumors with CV787 and docetaxel. Fig. 6B shows results from LNCaP prostate tumor xenografts treated with CV787 and docetaxel, both administered i.v. In the combination treatment group, animals received i.v. injections with docetaxel (5.0 mg/kg) on day 2, 5, and 8, after a single i.v. injection of CV787 (1 x 10$^{10}$ particles/animal) on day 1. Both CV787 and docetaxel appear to be effective in producing stabilization of tumor growth in the LNCaP mouse model, whereas a combination of the two produces a complete regression within 5 weeks (Fig. 6B). Analysis on fractional tumor volume, presented in Table 1, indicated a synergistic effect between CV787 and docetaxel in LNCaP xenografts. For example, on day 42, the CV787 and docetaxel combination group showed a 6.4-fold higher inhibition of tumor growth over an additive effect (Table 1).

To further investigate the dose range for CV787 treatment in combination with docetaxel, we fixed the dose of docetaxel at 12.5 mg/kg and varied the dose of CV787 from 1 x 10$^{8}$ and 1 x 10$^{9}$ to 1 x 10$^{10}$ particles/animal. Fig. 6C shows that treatment with CV787 alone or docetaxel alone resulted in tumor growth inhibition. How-

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**Table 1 Combination treatment with CV787 and taxane**

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<th>Treatment</th>
<th>Day$^b$</th>
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$^a$ FTV, fractional tumor volume, calculated as mean tumor volume experimental/mean tumor volume control.

$^b$ Day after tumors treated with agents.

$^c$ Mean FTV of CV787 x mean FTV of paclitaxel.

$^d$ Obtained by dividing the expected FTV by the observed FTV. A ratio of $>1$ indicates a synergistic effect, and a ratio of $<1$ indicates a less than additive effect.
ever, the combination of CV787 and docetaxel had the greatest effect of the treatments tested. Complete regression was achieved in the animals treated with docetaxel and CV787 at a dose of either $1 \times 10^{10}$, $1 \times 10^9$, or $1 \times 10^8$ particles. Synergy of antitumor activity was also evident using $1 \times 10^7$ particles/animal, but complete regression was not observed (data not shown). These changes were statistically significant by Student's t test for the comparison of combination treatment of CV787 and docetaxel with any of the vehicle, CV787 alone or docetaxel alone treatments, with no statistical difference between the three combination treatment groups. The complete response dose of CV787 alone is $1 \times 10^{11}$ particles/animal (7). Thus, the combination of CV787 and docetaxel produces a complete response with 1000-fold less virus, compared with the use of CV787 alone.

Virus replication within LNCaP tumors was documented by immunohistochemical staining of tumor sections using polyclonal antibodies to Ad5 (14). Fig. 7 contains representative tumor sections from four animals 9 days after treatment with either paclitaxel, CV787, or CV787 and paclitaxel. No evidence of virus replication was found in the tumors treated with either vehicle or paclitaxel, whereas evidence of necrosis and multifocal inflammation was observed in a small portion of tumors treated with paclitaxel (Fig. 7A, L). In the CV787-treated tumors, although positively stained cells were visible throughout the tumors, infected cells were predominantly located near the tumor vasculature (Fig. 7A, M). The most intriguing phenomena were in the samples treated with both the virus and paclitaxel. Although few virus-infected cells were detected, most of the cells in the sections were empty and virtually devoid of cellular content. The remaining cells were much smaller and appeared to have undergone a morphological change (Fig. 7A, R).

Tumor cells were also tested for apoptosis using the M30 Cytodeath detection kit, which recognizes a specific caspase cleavage site within cytokeratin 18 in early events of apoptosis. Three tumors from each group, CV787 alone, paclitaxel, or both CV787 and paclitaxel, were analyzed 9 days after the start of dosing (Fig. 7B). Few apoptotic cells were detected in the paclitaxel-treated tumor (Fig. 7B, L), although a significant number of apoptotic cells along the blood vessel were present in the CV787-infected tumors (Fig. 7B, M). However, combination treatment produced more apoptotic cells than in any of the other samples (Fig. 7B, R). In conclusion, the immunohistochemical analysis of CV787-treated tumors suggests that both virus replication-dependent cytotoxicity and apoptosis contribute to the antitumor effect of CV787 and taxane.

Finally, there are two other results of clinical significance. First, healthier animals, characterized by body weight, were observed in the combination treatment group as compared with groups treated with either agent alone (Table 2). Of particular interest is the transient weight loss caused by using docetaxel alone, from which animals are healthier than untreated control animals do (Table 2). Second, formal toxicology studies in BALB/c mice failed to show synergistic toxicity from the combined use of docetaxel and CV787 (data not shown).

Discussion

The treatment of human prostate metastatic cancer remains a formidable problem inasmuch as therapeutic advances have not significantly improved clinical outcome. The utilization of a replication-competent cytolytic adenovirus as a therapeutic modality shows much promise. In our laboratory, CV787 has been shown to be effective against human prostate tumors (7). However, total eradication of human LNCaP xenografts in our animal model required as much as one third of the lethal dose of virus (7, 14). Therefore, strategies to improve the efficacy of this form of treatment are desirable. In efforts to augment the cytotoxic activity of CV787, we have investigated a combination therapy using the taxanes paclitaxel and docetaxel.

A synergistic decrease of cell viability of LNCaP prostate cancer cells was observed when CV787 was combined with taxane. LNCaP cells cultured with paclitaxel or docetaxel for 24 h before or after infection with CV787 had significantly decreased viability than cells treated with either agent alone. LNCaP cells treated with taxanes exhibited a greater burst size of CV787, whereas no significant effect on viral growth kinetics was seen. No significant difference in the effectiveness of the combined therapy of taxane and CV787 infection was observed by varying the time of taxane administration, whereas varying the administration schedule of paclitaxel with Ad-p53 gene therapy can modulate the synergistic activity between these two agents in ovarian cancer (26).

In addition, combination treatment of CV787 with taxane did not alter the specificity of replication-mediated cytotoxicity. Cell viability assays indicated that CV787 in combination with taxane remains fully selective. This result was confirmed using the one-step growth curve. CV787 has been shown previously to replicate preferentially in PSA-producing human prostate cancer cells 10,000 times more efficiently than in non-PSA-producing cells (7). In the presence of paclitaxel or docetaxel in the culture medium, CV787 replicated to the same degree of specificity in the non-PSA-producing human cell lines versus PSA + cells.

A synergistic antitumor efficacy of combination therapy with CV787 and taxane was also observed in prostate LNCaP xenografts. Previous studies have demonstrated that tumors were eliminated within 6 weeks by a single i.v. administration of CV787 at a dose of $1 \times 10^{11}$ particles (7). Combination treatment of CV787 and taxane eliminated tumors within 4 weeks, whereas CV787 alone at the same dose of $1 \times 10^{10}$ particles/animal could only slow down tumor growth. Statistical analysis of the in vivo studies indicated that the CV787 and taxane combination group showed a significant synergy with a 6.4–9.2-fold higher inhibition of tumor growth over additive effect.

In combination with a 12.5 mg/kg dose of docetaxel, $1 \times 10^8$ particles of CV787 led to a complete elimination of tumors within 4 weeks. Thus, the dose of CV787 required for complete remission has been reduced by 1000 times from $1 \times 10^{11}$ to $1 \times 10^9$ particles. Thus, the combination of docetaxel with CV787 has increased the potential therapeutic window from 1 to 1000.

Investigations into the efficacy of virus-mediated gene therapy in combination with anticancer drugs have been published recently by other groups and demonstrated that paclitaxel had a synergistic or an additive effect when combined with Ad-mediated p53 gene therapy in several cancer models (3, 26). Cheon et al. (27) combined an Ad vector, which contained the HSV thymidine kinase (HSV-TK) gene driven by the osteocalcin promoter, with a low dose of methotrexate and demonstrated a significant increase in efficacy and prolonged survival in a murine osteosarcoma model (27). A replication-selective adenovirus, ONXY-015, in combination with 5-fluorouracil or cis-DDP was shown to have a greater effect than either individual modality and prolonged survival (28, 29). Furthermore, a synergistic efficacy was also observed in the combination of a tumor-specific HSV mutant (HSV-1716) with chemotherapeutic agents in human non-small cell lung cancer (30). Thus, the combination of virus-based therapy with chemotherapy for the cancer treatment appears to be promising.

From a clinical point of view, two additional observations are significant. First, healthier animals, characterized by body weight, were observed in the combination treatment group as compared with
Fig. 7. Representative immunohistochemical analysis of LNCaP tumors treated with either paclitaxel (L), CV787 (M), or CV787 and paclitaxel (R) at ×200. Nude mice that bore LNCaP xenografts were sacrificed 9 days after the start of treatment with CV787. A, Adenovirus-infected cells stain red as indicated by a filled arrow. B, Apoptotic bodies stain brown as indicated by the open arrow.
groups treated with either agent alone. Second, formal toxicology studies in Balb/C mice failed to show synergistic or additive toxicity from the combined use of docetaxel and CV787.

The mechanism(s) of synergistic activity in the combination of taxane with CV787 is unknown at this time; however, our experiments suggest a few hypotheses. First, taxane at the synergistic dose may be augmenting viral replication. It has been previously shown that a low concentration of paclitaxel (1–14 nM) increased the number of cells transduced by recombinant adenovirus 3–35% in a dose-dependent manner (26). Indeed, our data show that although the synergistic dose of paclitaxel or docetaxel did not alter virus replication kinetics (Fig. 2), the chemotherapy drugs slightly increased the burst size of CV787 in LNCaP cells. Secondly, CV787 may be augmenting the antitumor activity of taxane. EIA gene expression has been shown to increase cellular sensitivity to chemotherapeutic agents (31), and this enhanced sensitivity is partially caused by the induction of p53-dependent apoptosis by the E1A-induced sensitization of the cells (32). Recently, Ueno et al. (33) found that human ovarian cancer cells that were originally resistant to paclitaxel became paclitaxel-sensitive in E1A down-regulated HER-2/neu cells (33). In CV787, the E1A gene is intact and may be overexpressed in PSA-producing LNCaP cells. E1A is a potent inducer of p53 protein expression in infected cells (34), p53 levels may increase after infection, thereby increasing cell sensitivity to chemotherapy-induced apoptosis. Indeed, 2–8-fold more p53 protein was detected in the CV787 and docetaxel-treated LNCaP cells than that in the cells treated either with docetaxel alone or CV787 alone. This is consistent with the observation that more apoptotic cells were seen in the LNCaP tumors that received combination treatment than in tumors that received either agent alone (Fig. 7B). The actions of the two agents may be occurring at two distinct points in the same pathway. The activities of taxane and CV787 may be affecting distinct points in the same or different cell death pathways used in LNCaP cells. Further investigation of the possible mechanism(s) of synergistic activity in the combination of taxane with CV787 is under way.

Certainly, LNCaP mouse xenografts are likely to be more susceptible to docetaxel and CV787 chemotherapy than prostate cancer patients. For example, repeated injection of docetaxel can cure mice of LNCaP tumors3 (15) but cannot cure men of prostate cancer, and preexistent antibody to adenovirus can abrogate adenovirus-mediated antitumor activity (14).

In summary, we have developed a novel therapeutic strategy for the treatment of prostate cancer with a prostate cancer-specific adenovirus mutant, CV787, in combination with conventional chemotherapeutic agents. This combination therapy produced an additional therapeutic benefit over either individual modality. Low-dose taxane produced a synergistic effect with CV787 both in vitro and in vivo, which suggests an important possible neoadjuvant therapy for the treatment of prostate cancer. The combination of taxane and CV787 created a single dose curative in vivo therapeutic window in the LNCaP Balb/C nude xenograft model of over 1000:1.

3 Unpublished observations.
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Antitumor Synergy of CV787, a Prostate Cancer-specific Adenovirus, and Paclitaxel and Docetaxel

De-Chao Yu, Yu Chen, Jeanette Dilley, et al.

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