Molecular Chemotherapy and Chemotherapy: A New Front against Late-Stage Hormone-Refractory Prostate Cancer

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Abstract

Purpose: Stemming from its inherent heterogeneity, single-agent treatments are essentially ineffective against castration-resistant prostate cancer (CRPC). Thus, clinically relevant regimens that harness different modalities to maximize treatment efficacy without increasing cumulative toxicities are urgently needed. Based on this rationale, we investigated whether a novel combination of purine nucleoside phosphorylase–mediated, gene-directed enzyme-prodrug therapy (PNP-GDEPT) with docetaxel against CRPC has superior efficacy in comparison with individual treatments.

Methods: The in vitro cell growth inhibition in differentially treated murine and human CRPC cell lines was established using a cell-viability assay. The extent of synergy, additivity, or antagonism between treatments was evaluated using CalcuSyn statistical analyses. The local and systemic effects of docetaxel and/or PNP-GDEPT were tested in both immunodeficient and immunocompetent mice against human and murine CRPC tumors, respectively. Subsequently, immunohistochemical analyses and an evaluation of serum cytokine and serum toxicity profiles were conducted to characterize the differential host responses to treatment.

Results: The combined use of PNP-GDEPT and docetaxel led to strong synergistic cell killing in vitro. Compared with the individual modalities, a combination of the 2 led to a marked reduction in "local and distant" tumor growth in vivo, and importantly, with lowered doses and without additional toxicities. Immunomodulation was indicated by enhanced immune cell infiltration and altered serum cytokine levels. Furthermore, a lowering of T-helper type 2 cytokines, MCP-1, interleukin (IL)-4, IL-6, and IL-10 marked lower tumor burden and enhanced treatment efficacy.

Conclusion: PNP-GDEPT and docetaxel are a potent combination against CRPC in immunocompetent and immunodeficient settings; these outcomes have implications of translational potential for improved treatment and management of CRPC patients. 

Introduction

Treatment failure and poor quality of life are 2 primary issues that confront patients with castration-resistant prostate cancer (CRPC; ref. 1). After surgical resection and radiation therapy, castration or antiandrogen therapy is effective for recurrences (median survival: 30 months) but, eventually, CRPC, which is essentially incurable, develops. Furthermore, the age at diagnosis continues to decrease and, in men with high-grade PC, the younger men show relatively poor prognosis compared with older men (2). Clearly, it is vital to develop new therapies that are effective against CRPC yet cause minimal side effects.

CRPC is complex and multifocal (pathogenesis reviewed in ref. 3); advanced-stage metastatic PC is often considered as a group of diseases even within the same individual. Thus, various PC subpopulations respond uniquely to different therapies An obvious approach to overcome CRPC "robustness" would be to target its heterogeneity by developing rationale-based combinations of traditional and/or new alternative therapies that can activate divergent or complementary pathways of apoptosis. A synergy between such therapies would lead to lowered doses of each component when used in combination. This can significantly augment the quality of life and general clinical outcome, thus allowing improved management of patients with CRPC.

Recently, a tubulin/microtubule-targeting chemotherapeutic agent, docetaxel (4), was found to increase survival...
of approximately 40% of CRPC patients by a median of 2 months (5). This established docetaxel as the new standard of care for treating CRPC, both as a monotherapy and in combination with other drugs such as prednisone (6). However, the benefits are marginal with side effects that are not well tolerated, particularly in elderly patients (7). Consequently, its use in novel combinations with other modalities is being investigated (6).

To this end, molecular chemotherapy, engendered by gene-directed enzyme prodrug therapy (GDEPT)-based approaches have generated significant interest, albeit with suboptimal efficacy as monotherapy (6). Administered locally, GDEPT allows tumor-directed toxicity, including to tumors in distant locations. PC is particularly suitable; locally, GDEPT allows tumor-directed toxicity, including suboptimal efficacy as monotherapy (6). Administered approaches have generated significant interest, albeit with gene-directed enzyme prodrug therapy (GDEPT)-based

Consequently, its use in novel combinations with other modalities is being investigated (6).

Purine nucleoside phosphorylase–mediated GDEPT (PNP-GDEPT) offers a promising option; the PNP gene mediates intratumoral conversion of the systemically administered prodrug fludarabine phosphate (Fludara) to its toxic metabolites. These metabolites inhibit DNA and RNA synthesis (8) and, thus, effectively kill dividing and quiescent cells. Furthermore, through passive diffusion to surrounding cells, these lead to "local" and "distant" (metastases) bystander cell killing. In fact, PNP-GDEPT efficacy against late-stage PC has been previously shown in our laboratory in xenogeneic and syngeneic mouse models of local and metastatic CRPC (human PC-3 and murine RM1; refs. 9–11), and significantly, PNP-GDEPT prolonged survival rate of mice with transgenic adenocarcinoma of the prostate (TRAMP; ref. 12). Furthermore, host immune stimulation was implicated in these effects. Based on these findings, a dose-finding, phase I clinical trial to determine the safety and tolerability of Adenovirus (Ad)-mediated PNP-GDEPT has been approved in Australia (clinical trial number NCT00625430).

Based on their individual promise against CRPC and different modes of action, we hypothesize that a combination of PNP-GDEPT and docetaxel has the potential to target a wider repertoire of PC cells and thus lead to potent synergies against CRPC. If proven, the clinical translation of the outcome could be fast-tracked to the clinical setting (i) because both regimens are clinically relevant (docetaxel and Fludara are clinically proven with established pharmacokinetics; refs. 4, 13) and (ii) because of prospective superior targeting of local and systemic disease that occur at therapeutically lower doses in combination therapy.

Both PNP-GDEPT and docetaxel treatments may lead to dysregulated immune responses, as reflected in the involvement of immune cells (9–12), and/or altered cytokine profiles in docetaxel-treated patients (14). In particular, fluctuations in T-helper type (Th) 1/Th2 cytokines have been successfully exploited for noninvasive assessment of cancer progression and patient response in the clinical setting (15, 16). Surprisingly, similar assessments in preclinical studies are limited. Given the variation in cytokine profiles engendered by different therapeutics (17), establishing such parameters to monitor response to new regimens of therapy would accelerate the clinical translation of a positive outcome. We have previously shown, in evaluating a combination of cytotoxic deaminase–uracil phosphoribosyl transferase with interleukin (IL)-12 and IL-18 gene therapy against RM1 tumors, that lowering of serum IL-4 and monocyte chemotactic protein (MCP)-1 was consistently associated with lower tumor burden (local and systemic; ref. 18). Overall, the study indicated that a change in the Th2 cytokine/chemokines was a more reliable predictor of tumor burden or response to therapy. Thus, to enhance the robustness of this study and with a view to facilitate clinical translation of the findings, we have assessed the serum levels of a panel of CRPC-relevant Th2 cytokines in differentially treated mice and have correlated the findings with tumor growth and treatment responses.

Materials and Methods

Cell lines and mice

The human CRPC line, PC-3 (American Type Culture Collection; ATCC) was cultured in RPMI media supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin (GIBCO). PC-3M-Luc-C6 line (expresses luciferase gene; Caliper Life Sciences) was cultured similarly, except for the addition of gentamicin (50 μg/mL). Murine CRPC RM1 cells (Dr T.C. Thompson, Baylor College of Medicine, Texas) and A549 (ATCC), a lung adenocarcinoma line were cultured in Dulbecco’s modified Eagle
medium (DMEM; Gibco) supplemented with 10% FBS. Five- to 6-week-old male C57BL/6 or BALB/c nude mice were purchased from the Animal Resources Centre, Perth, Western Australia, and maintained at the Biological Resources Centre, University of New South Wales (UNSW), Australia.

Viral vectors
Replication-defective Ad vectors expressing cytomegalovirus (CMV) promoter-regulated green fluorescent protein (GFP; Ad/CMV/GFP) or PNP gene (Ad/CMV/PNP) were constructed, propagated, and titrated on the basis of manufacturer's instructions provided with the AdEasy Adenoviral Vector System (Stratagene).

Drugs
Docetaxel (Taxotere; Aventis Pharmaceuticals Inc.), carboplatin (Pfizer), and fludarabine phosphate (Fludara; Schering-Plough) were used.

Assessment of cytotoxicity of docetaxel in CRPC cell lines
Cells treated with different concentrations of docetaxel were assessed for cell viability using WST-1 assay at different times as described above.

Evaluation of efficacy of combination of PNP-GDEPT with docetaxel
Cells were infected with either Ad/CMV/PNP or Ad/CMV/GFP (conducted in triplicate in 96-well plates). After 48 hours, virus-containing media were replaced with Fludara- and/or docetaxel-containing media. The uninfected cells were treated with docetaxel. After 3 to 5 days, cell viabilities were assessed using WST-1 assay.

Clonogenic assays to assess cytotoxicity of different treatments
Three days after combination or individual treatments, cells were re-plated in 6-well plates. After 6 to 9 doublings, adherent cell colonies (>50 cells) were stained with crystal violet (0.5% in absolute methanol) and counted.

Evaluation of therapeutic interactions between modalities
The therapeutic interactions between docetaxel and PNP-GDEPT were analyzed using Calcusyn software (BioSoft) developed by Chou and Talalay (19). This method allows statistical evaluation of interactions among 2 or more drugs, based on the median-effect equation correlating drug and its effects, and is used to derive an accurate value of relative potencies of different drugs. The median-effect plot (based on the logarithmic form of Chou's median effect equation) forms the basis of quantification of synergism, summation, and antagonism of drug combinations: log(fraction affected/fraction unaffected) versus log (dose). A value called combination index (CI) is generated that helps quantify the interactions for mutually exclusive and nonexclusive drugs: A CI < 1 implies synergism (expected additive effect); CI = 1 implies additive effect; and a CI > 1 shows antagonism (expected additive effect) between drugs. A clinically significant value, "dose reduction index" (DRI), is calculated that allows prediction of the fold reduction in individual modality dose when used in combination in comparison to when used alone.

Animal experiments
All experimental protocols were conducted in accordance with the Animal Care and Ethics Committee guidelines of the UNSW, which is based on the Helsinki Declaration of 1975 as revised in 2000. In general, experimental mice were monitored and weighed every 2 to 3 days and humanely euthanized when they first showed signs of distress, appeared moribund, and/or lost 20% weight.

Evaluation of synergy between docetaxel and PNP-GDEPT in BALB/c nude mice
Details of this experiment are shown schematically in Supplementary Figure S1. PC-3M-Luc cells infected with Ad/CMV/PNP at an MOI of 10 pfus/cell were harvested after 24 hours and then infected (2 × 10^5 cells/50 µL PBS) s.c. into the flank of BALB/c nude mice. Mice were randomly divided into 4 different groups based on treatment with (i) vehicle, (ii) docetaxel, (iii) PNP-GDEPT, and (iv) docetaxel plus PNP-GDEPT. The next day onward, different groups of mice received Fludara (50 mg/m^2/d) intraperitoneally (i.p.) for 5 consecutive days and/or docetaxel i.v. at a dose of 10 mg/kg on days 1, 6, and 12. Tumors were measured twice a week using digital calipers as described (20). At necropsy, tumors and other organs (lungs and lymph nodes) were snap-frozen or paraffin embedded for immunohistochemical studies. Mouse sera were collected and stored at −80°C until analysis.

Bioluminescent imaging of PC-3M-Luc-C6 cells or tumors in mice
The luciferase expression in PC-3M-Luc-C6 cells was confirmed by live imaging of cells cultured with media

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containing 15 mg/ml luciferin (Xenogen Corp.) using the IVIS 200 imaging system (Xenogen Corp.) according to the manufacturer’s instructions.

For animal studies, BALB/C nude mice implanted with PC-3M-Luc-C6 tumors were administered luciferin i.p. (150 mg/kg) and were imaged under sedation (3% isoflurane) using the IVIS 200 imaging system at 10-seconds to 1-minute exposure times. Image analysis was carried out using Living Image software (Xenogen Corp).

**Evaluation of synergy between docetaxel and GDEPT in C57BL/6 mice**

The scheme for these experiments is shown (Supplementary Fig. S2). Orthotopic (intraprostatic; Iprost) RM1 cells (5 x 10^3) were surgically implanted in the subcapsular region of the prostate of C57BL/6 male mice as described (21). On day 5, Ad/CMV/PNP (10^9 pfu/cell) was injected into the prostate tumors, followed by i.v. injections of RM1 cells (5 x 10^5; day 6) to establish the lung pseudometastasis. The next day onward, Fludara or saline was administered i.p. daily at approximately 200 to 400 mg/m^2/mouse for 5 consecutive days. On days 7 and 12, docetaxel (12.5 mg/kg/d) or vehicle control injections were given i.v. At necropsy (day 16), tumors/ organs and sera were collected and stored as described above. The lungs were fixed in Bouin’s reagent to facilitate lung colony counting as described (18).

**Assessment of survival of tumor-bearing mice given different treatments**

C57BL/6 mice (n = 50) with intraprostatic RM1 tumors were treated with (i) Ad/CMV/PNP alone (control; n = 9), (ii) PNP-GDEPT (n = 10), (iii) docetaxel (n = 9), (iv) docetaxel plus Fludara (n = 9), and (v) GDEPT plus docetaxel (n = 13), starting on day 5 (Supplementary Fig. S2). Treated mice were monitored and weighed every 2 to 3 days. At necropsy, the tumors/other organs and sera were harvested and stored as described above.

**Toxicity analyses in treated mice**

To assess treatment-related toxicities, treated and untreated tumor-bearing mice were weighed before and, regularly, after treatments. Serum-based–toxicity analysis was conducted at SEALS pathology laboratory at Prince of Wales Hospital, NSW, Australia. Serum samples (150 μL) from treated mice were analyzed for renal and liver toxicity using urea, creatinine, alkaline phosphatase (ALP), and alanine aminotransferase (ALT) as standard markers. Values for age-matched healthy normal mice were used as reference standards.

**Cytokine profiles in treated mice**

Sera from mice (25 μL in mouse serum matrix) were analyzed (n = 4–5) using a Multiplex Mouse Cytokine/Chemokine Magna Bead Panel (Millipore) for murine Th2 cytokines/chemokines, IL-4, IL-6, IL10, and MCP-1 as described previously (22). Briefly, 2-fold serial dilutions of standards (10,000–3.2 pg/mL) were done, and the samples were incubated with a cocktail of antibody-immobilized beads overnight at 4°C; this was followed by incubation with biotinylated detection antibody cocktail and then incubation with streptavidin–phycoerythrin. Plates were analyzed (Bioplex; Biorad Laboratories) according to manufacturer’s instructions. Standard curves were generated using the 5-parameter logistic method and median fluorescence was evaluated using the software Bioplex Manager.

**Immunohistochemical analysis of orthotopic prostate tumors**

Tumors were frozen and acetone-fixed, and 5-μm sections were stained to evaluate apoptosis by *in situ* detection using the M-30Cytodeath Kit (Alexis Biochemicals), for infiltrating T lymphocytes (CD4^+ and CD8a^+) and macrophages (F4/80^+), and positively stained cells were scored by light microscopy as described (18, 20). The antibody details are listed in Supplementary Table S1.

**Results**

The combined effects of docetaxel and PNP-GDEPT were evaluated *in vitro* and *in vivo* using the following cell lines: (i) A murine RM1 cell line that is produced by activating the ras and myc oncogenes (23). RM1 cells express androgen receptors and yet display androgen independence (the most aggressive CRPC phenotype encountered in humans), forming aggressive tumors. The RM1 model has been successfully used in preclinical studies to evaluate different anticancer therapies against local and metastatic PCs (9, 11, 18, 20, 24). (ii) Human PC-3 cells that exhibit several characteristics of late-stage CRPC and provide a clinically relevant model to assess antitumor responses of various therapies (25, 26).

**CRPC cells are responsive to docetaxel and PNP-GDEPT treatments**

After establishing the optimal plating density for PC-3 and RM1 cells (Supplementary Fig. S3A), their dose–response to different modalities was assessed. The growth-inhibitory effects of docetaxel were found to be dose-dependent in both cell lines (Fig. 1A), with PC-3 cells showing approximately 5-fold greater sensitivity to docetaxel [inhibition constant (IC)_{50}: 0.57 ± 0.15 nmol/L; R^2 = 0.97] compared with RM1 cells [IC_{50}: 3.05 ± 1.21 nmol/L; R^2 = 0.95]. To evaluate the efficacy of PNP-GDEPT, the nontoxic dose of Fludara was determined to be 1 μg/mL in both cell lines (Supplementary Fig. S3B), and their differential permissivity to Ad-transduction was established using Ad/CMV/GFP (Supplementary Fig. S3C). At the MOI of Ad/CMV/GFP greater than 100 pfu/cell, PC-3 cells displayed approximately 4- to 6-fold greater permissivity to Ad-transduction relative to that noted in RM1 cells. Evaluation of Ad/CMV/PNP (+1 μg/mL Fludara)-transduced cells showed dose-dependent cytotoxicity and associated bystander effects in both cell lines (Fig. 1B). Correlating this with their Ad-permissivity, the cytotoxic effects (% cell
Figure 1. In vitro evaluation of synergy between PNP-GDEPT and docetaxel in CRPC PC-3 and RM1 cells. A, response of PC cells to docetaxel treatment. Dose-response curves for PC-3 and RM1 cells treated for 5 days with docetaxel concentrations ranging from 0.1 to 100 nmol/L are shown. Cell survival as percentage of vehicle control cells (cells treated with corresponding concentrations of polysorbate 80 and ethanol) is plotted against docetaxel concentrations using GraphPad Prism. Values represent a mean (±SEM) of 3 experiments. B, evaluation of cytotoxicity and bystander effects associated with PNP-GDEPT in PC cells. Cells were infected with Ad/CMV/PNP or Ad/CMV/GFP (control) at different MOI followed by prodrug treatment (Fludara 1 μg/mL). Cell viability was evaluated on day 5 by using WST-1 reagent. Graphs show changes in cell viability relative to control (percentage of control cells) for the 2 cell lines. These data show that bystander effects and resulting cytotoxicity increased with increasing doses of test virus. The cytotoxic effects were higher in PC-3 cells (top) versus those obtained in the RM1 cells (bottom). Values represent mean (±SEM) of 3 experiments. C, evaluation of cell growth inhibition by the combination of PNP-GDEPT and docetaxel in RM1 cells. Cells treated with different doses of docetaxel and/or PNP-GDEPT were analyzed for cell viability using WST-1 assay on day 5. Graphs representing inhibition of cell growth were plotted as a function of increasing doses of docetaxel and PNP-GDEPT. Most drug combinations led to significantly greater cell growth inhibition than that achieved with either treatment alone. Values represent mean (±SEM) of 3 independent experiments. Similar results were obtained for PC-3 cells (data not shown). D, analysis of combined drug effects of docetaxel and PNP-GDEPT in PC cells. CI values for different combinations of docetaxel and PNP-GDEPT against the fraction affected (F_a) generating CI/F_a plots by using the Chou and Talalay method. Synergistic effects are indicated for both PC-3 (I) and for RM1 cells (B), as most of CI values are significantly less than 1 at almost all drug combinations.
death relative to untreated controls (± SEM) were significantly higher in PC-3 cells (68% ± 4 cell killing) compared with that in RM1 cells (25% ± 4 cell killing) at MOI of 300. Calculation of the effective dose (MOI of Ad/CMV/PNP + Fludara) to achieve 50% cell killing (ED$_{50}$) indicated a greater potency of Ad-PNP-GDEPT against PC-3 cells (150 ± 8) than that against RM1 cells (600 ± 15) with no noticeable toxicity upon treatment with Ad/CMV/GFP + 1 µg/mL Fludara in both cell lines. Furthermore, "bystander cytotoxic effects" were clearly achieved as shown by a greater percentage of cell killing than the percentage of transduced cells (at an MOI of 500, although only 8% and 35% of RM1 and PC-3 cells were infected, cell killing of ~45% and >80% was noted in RM1 and PC-3 cells, respectively; Supplementary Fig. S3C).

### Table 1. Effects of combining PNP-GDEPT and docetaxel against CRPC cells: a comparison between efficacies of drug combinations at different ratios in CRPC cells

<table>
<thead>
<tr>
<th>Modalities (ratios)</th>
<th>Cell line</th>
<th>CI value$^a$</th>
<th>$R^2$</th>
<th>Effect$^b$</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>ED$_{50}$$^c$</td>
<td>ED$_{75}$</td>
<td>ED$_{90}$</td>
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<tr>
<td>1:1</td>
<td>PC-3</td>
<td>0.6924</td>
<td>0.4648</td>
<td>0.3120</td>
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<td></td>
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<tr>
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<td>0.4329</td>
<td>0.3485</td>
</tr>
<tr>
<td></td>
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<td>0.4309</td>
<td>0.3012</td>
</tr>
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<tr>
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<td></td>
<td>RM1</td>
<td>0.6634</td>
<td>0.5185</td>
<td>0.3284</td>
</tr>
</tbody>
</table>

$^a$CI values generated when drugs were plotted as constant ratios 1:1 (e.g., IC$_{25}$ of docetaxel with IC$_{25}$ of carboplatin.

$^b$Effective dose, which can result in 50%, 75%, and 90% of cell killing.

$^c$The linear correlation coefficient $r$ of the median-effect plot. The acceptable range of "R" values varies with the type of system used; enzyme or receptor systems ($r > 0.96$), tissue culture systems ($r > 0.90$); and animal experiments ($r > 0.85$).

$^d$Combination effects: CI values from 0.90 to 1.10 signify additivity (•); CI from 0.7 to 0.85 indicate moderate synergism (••); CI from 0.30 to 0.70 suggests synergism (•••); CI from 0.10 to 0.30 shows strong synergism (••••••); and CI from 0.01 to 010 indicates very strong synergism (••••••••••).

PNP-GDEPT and docetaxel interact synergistically against CRPC cells

The nature of the interactions between PNP-GDEPT and docetaxel against the 2 cell lines was evaluated at different doses of both agents using cell-viability and clonogenic assays to assess short- and long-term responses, respectively. In both cell lines, a significant dose-dependent inhibition of cell growth was noted using either modality, which was further enhanced when the treatments were combined (Fig. 1C; data for PC-3 cells is not shown). This was reaffirmed by clonogenic assays; a reduction in percentage cell survival by approximately 6- to 7-fold was noted when PC-3 cells were treated with a combination of 2 modalities versus either alone at a predetermined ED$_{50}$ dose (Supplementary Fig. S4). Statistical analysis of these interactions by CalcuSyn software (BioSoft) for PC-3 and RM1 showed that the combined efficacy of PNP-GDEPT and docetaxel was due to synergistic interactions at most of the combination ratios tested (Table 1). In spite of differences in their susceptibility to Ad-PNP-GDEPT, similar levels of synergy were obtained for PC-3 (CI: 0.46 to ED$_{75}$; R-value: 0.99) and RM1 cells (CI: 0.39 at ED$_{75}$; R-value: 0.98) when docetaxel and PNP-GDEPT were combined at a 1:1 ratio. Similar synergies were noted when combinations were evaluated at other ratios (e.g., 1:2, 2:1, 1:4; ref. Table 1). Based on these data, calculation of DRI values showed that, in combination, much lower doses of docetaxel and PNP-GDEPT (up to 6- to 8-fold for docetaxel and 6-fold for PNP-GDEPT) were predicted to achieve the desired effect against both PC-3 and RM1 cells (Table 2).

Combination of PNP-GDEPT and docetaxel significantly reduces the growth of PC-3M-Luc-C6 xenografts in nude mice

Given the positive in vitro outcomes, effects of combination of docetaxel and PNP-GDEPT on s.c. growth of PC-3M-luc-C6 tumors was determined (Supplementary Fig. S1). The PC-3M-Luc-C6 line is a more aggressive variant of PC-3 and was permissive to Ad-transduction; cells could be detected by bioluminescent imaging (Supplementary Fig. S5). Tumor growth in response to different treatments was monitored by bioluminescent imaging (mean tumor bioluminescence; relative to tumor size; MTB) or by caliper measurement (mean tumor volume; MTV; Fig. 2). On day 35, however, a decrease in MTB was noted in docetaxel-treated (3.61E + 009 ± 1.124E + 009; P < 0.001) and PNP-GDEPT-treated (1.90E + 009 ± 4.55E + 008; P < 0.001) mice; the decrease in MTB in mice treated
with combination therapy \( (3.11 \pm 0.008 \times 2.32E + 008; P < 0.001) \) was significantly higher (Fig. 2A and B) relative to controls \( (3.81 \pm 0.10 \times 7.92E + 009). \) These trends correlated with those noted using caliper measurements (Fig. 2C).

| Table 2. Effects of combining PNP-GDEPT and docetaxel against CRPC cells: dose reduction index values when GDEPT and docetaxel were used at 1:1 combination against PC-3 and RM1 cells |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Fraction affected               | Drug alone \( (\times IC_{50}) \) | Dose reduction index \( (\times IC_{50}) \) | Fraction affected               | Drug alone \( (\times IC_{50}) \) | Dose reduction index \( (\times IC_{50}) \) |
| GDEPT                           | Docetaxel                        | GDEPT                           | Docetaxel                        | GDEPT                           | Docetaxel                        |
| 0.1                             | 0.26                             | 1.33                            | 0.1                             | 0.96                            | 0.12                             |
| 0.25                            | 0.98                             | 1.99                            | 0.25                            | 2.27                            | 0.51                             |
| 0.50                            | 3.74                             | 2.95                            | 0.5                             | 5.38                            | 2.11                             |
| 0.75                            | 14.20                            | 4.39                            | 0.75                            | 12.75                           | 8.80                             |
| 0.9                             | 53.90                            | 6.53                            | 0.9                             | 30.16                           | 36.54                            |
| Overall, mice given combination therapy showed the best treatment efficacy in terms of rate of tumor growth and size \( (341 \pm 123; P < 0.001) \) in comparison with docetaxel \( (1,003 \pm 165; P < 0.001) \), PNP-GDEPT \( (761 \pm 145; P < 0.001) \), or

Figure 2. Effects of combination therapy on s.c. PC-3M-Luc-C6 tumors in BALB/c nude mice: Nude mice were injected s.c. with PC-3M-Luc-C6 cells infected with Ad/CMV/PNP at an MOI of 10 such that only approximately 10% to 12% of the cells were expressing. The prodrug Fludara was given i.p. at 75 mg/kg for 5 days and docetaxel was given as 10 mg/kg thrice as described in Supplementary Figure S1. Mice induced with Ad/CMV/GFP-expressing tumors served as the controls. The growth of luciferase-expressing tumors was monitored by imaging using Xenogen IVIS Lumina and by measuring the tumors using calipers regularly every week. A, graph shows the light emission [photon-flux (photons/s/cm²/sr); steradian (sr) is a unit of solid angle) measured on day 35 in mice given different treatments. B, graph shows the tumor volumes (caliper measurements) at different times during the course of experiment. C, the visual bioluminescent images of mice with tumors given different treatments on week 5. Mice given the combination treatments showed the best efficacy and rate of growth, and size of tumors was minimal in these mice.
control mice (1,645 ± 179). Importantly, there was no detectable tumor growth in 8 mice in the combination group with only 3 of 11 mice displaying visible tumors. Finally, weight loss, reflecting treatment-related toxicity, did not exceed 10% in any group (Supplementary Fig. S6).

Combination of docetaxel and PNP-GDEPT significantly enhances efficacy against RM1 tumors in immunocompetent mice

Having showed the benefit of combination therapy against PC-3M-Luc-C6 xenografts, evaluations were conducted in syngeneic RM1 models of orthotopic and lung tumors (Supplementary Fig. S2). To show unequivocally the potential of combination therapy, PNP-GDEPT and docetaxel treatments were given at suboptimal doses. These doses were determined in dose-optimization experiments wherein mice with intraprostatic and lung RM1 tumors were either treated with different doses of PNP-GDEPT (by changing doses of Fludara; range: 100–300 mg/m²/d; Supplementary Fig. S7) or with docetaxel at 3 different doses (range: 10–20 mg/kg/d; Supplementary Fig. S8). In the former, only mice given Fludara at 300 mg/kg showed a significant reduction in local tumor growth and lung pseudometastases (Supplementary Fig. S7). In docetaxel-treated mice, local tumor reduction was noted at all doses tested, with marginally improved efficacy at higher doses of 15 and 20 mg/kg (Supplementary Fig. S8). However, docetaxel was not effective against RM1 lung pseudometastases at all doses tested. For combination experiment, a suboptimal dose of PNP-GDEPT (Fludara at 250 mg/m²/d) and docetaxel at 12.5 mg/kg/d were chosen.

Enhanced efficacy of combination treatment against local PC growth

Data clearly showed the enhanced efficacy of combining PNP-GDEPT and docetaxel on local PC growth versus that obtained with individual treatments or docetaxel plus Fludara (Fig. 3A). Comparisons of treatment groups showed a significant difference in tumor growth (P < 0.05); there was negligible to very little tumor growth in 5 of 9 combination-treated mice compared with 3 of 9 mice treated with docetaxel, 1 of 9 mice given PNP-GDEPT alone, and 1 of 8 given docetaxel plus Fludara.

Enhanced efficacy of combination treatment on growth of RM1 lung pseudometastases

To evaluate systemic effects, the effects of different treatments were assessed against RM1 lung pseudometastases. Mice treated with PNP-GDEPT plus docetaxel showed a greater reduction in lung colonies versus those receiving PNP-GDEPT, docetaxel, or docetaxel/Fludara; in combination-treated mice, less than 50 colonies were noted in 4 of 9 mice compared with 1 of 9 in PNP-GDEPT-treated mice (Fig. 3B). Results of lung-mass measurements reinforced these trends (Fig. 3C). Again, as observed in previous experiments, treatment with docetaxel alone had no effect on lung tumor growth (Supplementary Fig. S8).

Combination regimen does not lead to added toxicities in mice

Next, we estimated whether combining PNP-GDEPT with docetaxel led to cumulative toxicities in treated mice. Ad-vectors cause hepatotoxicity in vivo and Fludara and docetaxel are primarily processed through the renal/urinary tract and liver, respectively. An assessment of the biochemical markers of kidney function (urea and creatinine) and markers of liver function (ALP and ALT) in the sera of mice showed no significant differences between treated groups versus untreated age-matched healthy mice (Supplementary Table S2). This indicated a lack of renal toxicity or hepatotoxicity due to any of the treatments. In addition, regular monitoring of mice for symptoms such as lethargy, poor responsiveness to stimulus, and weight loss showed no differences between groups (Supplementary Fig. S9).

Combination of PNP-GDEPT with docetaxel improves the survival of RM1 tumor-bearing mice

Finally, the effect of combination therapy on mouse survival was assessed. Kaplan–Meier’s survival analysis indicated a statistically significant survival advantage (by 60%) in mice given the combination of PNP-GDEPT and docetaxel in comparison to mice given control treatment (P < 0.0001; Mantel–Cox rank test; Fig. 3D). On day 25, the percentage of surviving mice was more than 50% in combination-treated mice versus only 10% in the PNP-GDEPT–treated group and none in the other treatment groups. The median survival of combination-treated mice was 25 days versus 19, 18, and 15 days for those given PNP-GDEPT, docetaxel + Fludara, or docetaxel or virus alone, respectively.

Combination therapy results in increased apoptosis and immune infiltration in RM1 tumors

The success of anticancer therapies is dependent upon apoptotic tumor cell death and is augmented by a favorable immune response. An immunohistochemical assessment of immune cell infiltration in prostate tumors revealed that the combination therapy led to a greater tumor infiltration by cytotoxic T lymphocytes (CD8a+; P = 0.13; 2.25-fold) and macrophages (F4/80+; P = 0.04; 1.3-fold) versus the controls (Fig. 4; Supplementary Table S3). This was substantially greater than infiltration observed in tumors from mice given either docetaxel or PNP-GDEPT. Although not significant, a higher level of Th cell (CD4+) infiltration was noted in combination-treated tumors compared with those treated with docetaxel or PNP-GDEPT (P = 0.16). Furthermore, a significant increase in apoptotic cells (M30+) was noted in combination-treated tumors (P = 0.02; Fig. 4; Supplementary Table S3) versus those from other groups. Surprisingly, in spite of notable tumor reduction, almost negligible numbers of apoptotic cells were noted in tumors treated with either PNP-GDEPT or docetaxel alone.
The treatment efficacy is associated with reduction in serum Th2 cytokines in mice

After the superior efficacy of the combination was established in 3 different models, we investigated its effect on Th2 cytokine profiles. Such monitoring will be valuable to manage patients when this regimen is applied in the clinical setting. We focused on Th2 cytokines only because, previously, we observed that changes in Th2, and not Th1, cytokines better indicated effects on tumor burden/respons to therapy. A panel of Th2 cytokines, MCP-1, IL-4, IL-6, and IL-10 that are implicated in prostate cancer progression and pathology were selected (27–34). Sera analyses showed a reduction in all mice given the combination regimen (Fig. 5A) in comparison to either treatment given alone. In particular, MCP-1, IL-4, and IL-6 levels correlated with tumor burden and were lowered markedly in mice with tumors weighing less than 1 g and lung colony numbers less than 250 versus those with prostatic tumors.

Figure 3. Effects of combination of PNP-GDEPT and docetaxel treatments on RM1 tumor growth in C57BL/6 mice. Tumors grown intraprostatically in C57BL/6 mice were injected with 10^9 pfu of Ad/CMV/PNP and then systemically treated with Fludara at 250 mg/m^2/d for 5 days. Docetaxel was administered at 12.5 mg/kg on days 7 and 12. At necropsy, day 16, the harvested tumors were weighed/measured; lungs were weighed and stored for lung colony counting. Other organs and sera were stored for other analyses. The graphs show the effects of different treatments on local prostate tumor growth (A), lung pseudometastases as evaluated by lung colony counts (B), and lung mass measurements (C). The survival of RM1 intraprostatic tumor-bearing C57Bl/6 mice given different treatments was assessed through posttreatment monitoring of these mice until euthanasia (loss of >20% weight or loss of condition). Kaplan–Meier analyses of survival show the best survival for combination-treated mice with 60% better survival compared with the control mice (D).
Weighing more than 1 g and more than 250 lung colony numbers (Fig. 5B). (Note: Using 2 measures of tumor burden for grouping ensured that none of the mice were within the 10% margin.) These data were reaffirmed from serum cytokine profiling of mice from GDEPT or docetaxel dose-optimization experiments (Supplementary Figs. S7D and S8D). In particular, MCP-1 and IL-6 levels reflected changes in intraprostatic tumor burden. The lung colony numbers were not markedly different between mice given different doses in both experiments (Supplementary Figs. S7C and S8C, with the exception of mice given GDEPT 300 vs. control mice). A reduction in IL-4 levels was specifically observed in mice given docetaxel treatment (Supplementary Fig. S8).

**Discussion**

Complementary therapeutic modalities that widen the target-cell repertoire but do not enhance combined toxicities have the best prospects of success against CRPC and for quick clinical translation. In this study, we have unequivocally showed synergistic interactions between Ad-mediated PNP-GDEPT and docetaxel against human and murine CRPC (RM1 and PC-3 cells) *in vitro*, in immunodeficient and immunocompetent tumor-bearing mice. The fact that this combination could target a wider cell repertoire was obvious from the strong *in vitro* and *in vivo* synergies noted against human PC-3 and murine RM1 cells with different levels of Ad-permissivity and docetaxel-sensitivity. In particular, synergies against Ad-refractory and docetaxel-resistant RM1 tumors were impressive, notably at suboptimal doses. More importantly, there was no evidence of additive toxicity with no changes to liver and kidney function and mouse weights (Supplementary Figs. S6 and S9; Supplementary Table S2). The clinical relevance of these data was corroborated by the DRI values that indicated up to 6-fold reduction in therapeutic doses of individual therapies when used in combination. Such reduction in therapeutic doses could significantly reduce side effects and improve patient management.

Enhanced involvement of apoptosis and immune stimulation (*immune cell infiltration, alteration in serum cytokine profiles, etc.*) when the modalities were combined, indicated generation of systemic antitumor immune responses and correlated with enhanced long-term survival benefits in combination-treated mice (Fig. 3D); on day 20, 90% of combination-treated mice were surviving versus none in the control-treated group (*P* < 0.0001). Although, the immune system was implicated, effective synergy in immunodeficient mice suggested that this combination therapy may be successful in severely immunocompromised patients (as is often the case in patients undergoing chemotherapy); at suboptimal doses of PNP-GDEPT (10%–12% Ad/CMV/PNP transduction) and docetaxel (10 mg/kg every 4 days vs. 12.5 every 3 days used in our previous experiments), there was
no tumor growth in approximately 70% of the mice in the combination-treated group. Thus, interactions other than those involving the adaptive immune system may be involved, for example, docetaxel-mediated enhancement of Ad-transduction or Ad-mediated enhancement of docetaxel sensitivity may have contributed to the synergistic enhancement of combined cytotoxicity (35, 36).

The efficacy of PNP-GDEPT against both cell types correlated with our previous results (25, 37). Given the potential toxicities of systemically administered Fludara (38), dose optimization was done on the basis of variable Fludara doses rather than local Ad/CMV/CMV dose, with the highest dose being 2-fold lower than that used in our previous studies (600 mg/m²/d for 5 days; refs. 11, 12, 39). Although, the Fludara dose in nude mice (75 mg/m²/d, 5 days) is approximately 3 times and in C57BL/6 mice (highest dose: 300 mg/m²/d, 5 days) is approximately 12 times, greater than that given to humans (25–30 mg/m²/d, 5 days; ref. 40), it is well within the acceptable physiologic range because mice have 45 times higher Fludara-tolerance than humans (41). The synergy developed with docetaxel when using low doses could have beneficial effects in the clinical setting, particularly since one of the major side effects of docetaxel is neutropenia (loss of circulating white blood cells) and Fludara is associated with immunosuppression due to lymphopenia (abnormally low levels of lymphocytes).

Surprisingly, at all doses tested, systemic docetaxel was effective only against local tumor growth with no effect on growth of lung pseudometastases. The suboptimal doses (expected to yield intermediate effects based on our assessments) of 10 and 12.5 mg/kg chosen for combination studies in nude and C57BL/6 mice, respectively, are clinically relevant, leading to plasma concentrations within the range achieved for humans administered 75 to 100 mg/m² of docetaxel (42). The lack of systemic efficacy could be due to (i) resistance of RM1 cells to docetaxel, (ii) toxicity of docetaxel to circulating immune cells, and (iii) short half-life of docetaxel in mice; a lowering of plasma levels below the toxic threshold after 45 minutes (42) between doses (n = 2, every 4 days) was noted. In contrast, its efficacy against the local tumors could be due to better survival of infiltrating immune cells due to reduced docetaxel penetration within the solid tumor mass (immune cells were noted in these tumors). Other factors such as greater sensitivity of stromal cells to docetaxel (43) may have enhanced its localized antitumor effects.

Interestingly, the systemic efficacy of the combination was far greater than that of either treatment administered alone (Fig. 3), suggesting a positive interaction between the two. Given the promising synergies obtained in this study, interactions between PNP-GDEPT metabolites and docetaxel need to be further explored to better understand the mechanisms underlying the synergies for future application.

The immunomodulatory effects of docetaxel, Fludara, and Ad-vectors are well established. That inflammation leads to cancer development and progression is now evident. Although serum cytokines levels typically reflect the specific immune status of the host, a shift of the cytokine balance to Th2 has been shown to be associated with cancer progression (44, 45). On this premise and to further test our previous finding that Th2 cytokines are a reliable predictor of tumor burden and treatment efficacy, we undertook an analysis of a panel of Th2 cytokines, MCP-1, IL-4, IL-10, and IL-6 that are implicated in prostate cancer progression, including development of
androgen-refractory phenotype \((27, 29–31, 46–50)\). In agreement with our previous findings \((22)\), the reduced levels of serum Th2 cytokines, MCP-1, IL-4, and IL6 profiles (Fig. 5; Supplementary Figs. S7D and S8D), correlated with treatment efficacy and lower tumor burden (local and lung metastastases). This clearly established the promise of this panel to monitor tumor burden and response to treatment specific to this regimen.

There are few reports of the combined use of gene therapy and chemotherapy for CRPC treatment and, therefore, results from this study hold a definite promise for targeting PC heterogeneity and thus improving clinical outcomes with potential for customized patient management. The fact that cytoreductive gene therapy and chemotherapy is a clinically attractive and tangible option against CRPC is reinforced by the success of an oncolytic Ad CV787 combined with docetaxel and paclitaxel that has already crossed a number of clinical hurdles \((36)\).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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