In vitro and in vivo Responses of Advanced Prostate Tumors to PSMA ADC, an Auristatin-conjugated Antibody to Prostate-specific Membrane Antigen

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Abbreviations: ABC: ATP-binding cassette; ADC: antibody-drug conjugate; Bmax: maximum binding capacity; FBS: fetal bovine serum; mAb: monoclonal antibody; MMAE: monomethylauristatin E; PSMA: prostate-specific membrane antigen; TBST: tris-buffered saline-Tween 20; vc: valine-citrulline; ZJ24: N-[N-((S)-1,3-dicarboxypropyl)carbamoyl]S-[^3H]-methyl-L-cysteine

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ABSTRACT

Prostate-specific membrane antigen (PSMA) is a membrane protein that is overexpressed manifold in prostate cancer and provides an attractive target for therapy. PSMA ADC is an antibody-drug conjugate (ADC) that consists of a fully human anti-PSMA monoclonal antibody conjugated to monomethylauristatin E through a valine-citrulline linker. In this study, the antitumor activity of PSMA ADC was evaluated against a panel of prostate cancer cell lines in vitro and in a novel in vivo model of taxane-refractory human prostate cancer. In vitro cell killing was efficient for cells with abundant PSMA expression (>10⁵ molecules/cell; IC₅₀ ≤ 0.022 nM) and 1,000-fold less efficient for cells with undetectable PSMA (IC₅₀ > 30 nM). Intermediate potency (IC₅₀ = 0.80 nM) was observed for cells with ~10⁴ molecules of PSMA per cell, indicating a threshold PSMA level for selective cell killing. Similar in vitro activity was observed against androgen-dependent and -independent cells that had abundant PSMA expression. In vitro activity of PSMA ADC was also dependent on internalization and proper N-glycosylation/folding of PSMA. In contrast, less potent and non-selective cytotoxic activity was observed for a control ADC, free monomethylauristatin E and other microtubule inhibitors. PSMA ADC demonstrated high in vivo activity in treating xenograft tumors that had progressed following an initial course of docetaxel therapy, including tumors that were large (>700 mm³) prior to treatment with PSMA ADC. This study defines determinants of antitumor activity of a novel antibody-drug conjugate. The findings here support the clinical evaluation of this agent in advanced prostate cancer.
MAIN TEXT

Introduction

Prostate cancer is the most common non-skin cancer in men in most Western populations and it kills more than 200,000 men annually worldwide (1). Although conventional treatments, such as surgery, radiation and androgen suppression, are effective, many patients experience disease recurrence and ultimately succumb to their disease. Hormone-refractory prostate cancer is particularly difficult to control and many treatment protocols include a chemotherapeutic agent. An important consideration is the limited and transient response of prostate cancer to systemic chemotherapy and its potential toxicity. There is an urgent need for additional therapies.

Chemoresistance mechanisms are poorly defined in prostate cancer (2, 3). Docetaxel resistance has been generated in vitro (4-8); however, resistance pathways vary depending on the cell line and selection conditions. In addition, clinical resistance to taxanes has been linked to changes in tumor microenvironment as well as tumor cells (3). Additional preclinical models are needed to better assess the potential utility of new agents against prostate tumors that are refractory to standard therapies.

Monoclonal antibody (mAb) therapy for oncology has grown tremendously in the past decade (9-12). Currently, seven unconjugated mAbs and two radiolabeled mAbs are approved for cancer treatment in the United States with many more in development. However, mAbs to many tumor-associated antigens show limited activity in unmodified form. In such cases, mAbs can be covalently linked to a cytotoxic drug as a potential means to selectively deliver the drug to neoplastic cells while reducing toxicity towards healthy tissues. Antibody-drug conjugates (ADCs) hold increasing promise due to
continuing advances in cancer proteomics, antibody engineering, and drug-linker chemistries (9, 10, 13-15). Newer ADCs have demonstrated substantial clinical activity as single agents even in heavily pretreated patients (11, 16).

Prostate-specific membrane antigen (PSMA) is an attractive target for the treatment and detection of prostate cancer. It is a type II transmembrane protein with a short intracellular domain (amino acids 1-18), a transmembrane domain (amino acids 19-43) and a large extracellular domain (amino acids 44-750) (17-19). PSMA is expressed in nearly all prostate cancers, and expression is highest in poorly differentiated, metastatic, and hormone-refractory cases (17, 20-22). PSMA also is expressed in a variety of tumor, but not normal, vascular endothelium, which further broadens its potential utility as a therapeutic target (23-25). The cytoplasmic tail of PSMA mediates its internalization both in the presence and absence of mAbs (26, 27). With its abundant and restricted expression in tumors, its membrane location and rapid internalization, PSMA has characteristics desired in a target for ADC therapy.

Monomethylauristatin E (MMAE) is a synthetic dolastatin 10 analog that potently blocks tubulin polymerization. Dose-limiting toxicities of neutropenia, myalgia, and constipation have been reported for dolastatin 10 (28, 29), and drugs in this class may be more useful when selectively directed to cancer cells. An example of such targeted drug delivery is provided by brentuximab vedotin (SGN-35), an anti-CD30 mAb conjugated to MMAE through a valine-citrulline (vc) dipeptide linker. Brentuximab vedotin has shown promising tolerability and activity in the treatment of relapsed or refractory Hodgkin lymphoma and systemic anaplastic large cell lymphoma (11, 30).
PSMA ADC (Figure 1) comprises a fully human anti-PSMA mAb conjugated to vcMMAE (31). PSMA ADC is designed to selectively bind PSMA-expressing cells, internalize via the endocytic pathway, and release a cytotoxic dose of MMAE. The agent has entered phase 1 testing in patients with metastatic prostate cancer that has progressed following androgen-deprivation and taxane therapy. In the ongoing study, antitumor activity has been observed as decreases in prostate-specific antigen, circulating tumor cells and/or bone pain at generally well tolerated doses (32).

To further elaborate the antitumor spectrum of this agent, we report here the in vitro activity of PSMA ADC against androgen-dependent and -independent prostate cancer cell lines with varying levels of PSMA expression. We also describe the efficacy of PSMA ADC in a novel in vivo model of docetaxel-refractory prostate cancer. Overall, our findings provide insight into the potential utility of this targeted therapy in advanced prostate cancer.

**Materials and Methods**

**Cell culture** Prostate cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). LNCaP, C4-2, CWR22rv1, and PC3 cells were maintained in RPMI1640 (Mediatech Inc., Manassas, VA) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS; United States Biochemicals, Cleveland, OH). DU145 cells were maintained in DMEM (Mediatech Inc., Manassas, VA) supplemented with 2 mM L-glutamine and 10% FBS (United States Biochemicals). MDA PCa2b cells were grown in F-12K Medium (ATCC) supplemented with 20% FBS, 25 ng/mL cholera toxin,
10 ng/mL mouse epidermal growth factor, 0.005 mM phosphoethanolamine, 100 pg/mL hydrocortisone, 45 nM selenious acid and 0.005 mg/mL bovine insulin.

**Cytotoxic agents and antibodies** Paclitaxel and docetaxel were purchased from Sigma-Aldrich (St. Louis, MO). PSMA ADC, control ADC (human IgG conjugated to vcMMAE), and MMAE were prepared as described (31). Anti-PSMA mAbs J591 and GCP.04 were gifts from Dr. Neil Bander (Weill Cornell Medical College, New York) and Dr. Jan Konvalinka (Academy of Sciences, Czech Republic, Prague), respectively.

**Transfection of PC3 cells** PSMA point mutants with altered internalization (L5A) and N-linked glycosylation (N638A) have been described (33, 34). DNA (5 μg) was precipitated with Pires-puro (0.5 μg) and transfected into PC3 cells using Lipofectamine 2000 (Invitrogen, Calsbad, CA). Stable clones were selected with 1 μg/mL puromycin.

**Extraction of cell membrane** One 175-cm² flask of harvested cells was diluted with 10 mL of 50 mM Tris (pH 7.5), Dounce homogenized, and centrifuged at 3,000g at 4°C for 5 min. Supernatant was collected and ultracentrifuged at 70,000g at 4°C for 35 min. The pellet was then washed and homogenized in Tris buffer. Protein concentration was measured by BCA assay (Thermo Scientific, Rockford, IL).

**Western blotting** Cell membrane extract (20 μg protein) was resolved by 7.5% bis-acrylamide reducing SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 5% milk in Tris Buffered Saline-Tween 20 (TBST) for 1h at room temperature. PSMA was detected with mAb J591 or GCP.04 at ~0.2 μg/mL for 1h followed by incubation with HRP-goat-anti-mouse IgG antibody (1:5000 dilution) for 1h. After 3 TBST washes, blots were visualized by chemiluminescence.
Folate hydrolase assay  Cell membrane extract (2 μg) was combined with 5 nmol of methotrexate diglutamate (MTXGlu₂) (Schircks Laboratories, Jona, Switzerland). The reaction mixture was adjusted to 100 μL with 50 mM Tris (pH 7.5), incubated at 37°C for 1h and stopped with 100 μL 0.5M Na₂HPO₄. Reaction products were quantified by reversed-phase HPLC (33).

Binding of cell lines to N-[(S)-1,3-dicarboxypropyl]carbamoyl]-S-[³H]-methyl-L-cysteine (³H-ZJ24) Cells (5×10⁵) were incubated with ³H-ZJ24 (GE Healthcare Life Sciences) in a total volume of 200 μL of 50 mM Tris (pH 7.5) for 1h at 37°C. The mixture was centrifuged at 3,000g for 5 min at 4°C to separate bound and free ³H-ZJ24. The supernatant was removed, and the cell pellet was washed three times with 500 μL of cold Tris buffer. Four milliliters of ECOLUMNETM scintillation cocktail (MP Biomedicals, Costa Mesa, CA) was added, and radioactivity was counted. Non-specific binding was determined using the same method in the presence of 0.1 mM phosphonomethyl pentanedioic acid. Data were analyzed using GraphPad Prism 3.0.

In vitro cytotoxicity assay  Cytotoxicity was measured using the CellTiter 96 Aqueous Cell Proliferation Assay (Promega, Madison, WI). Briefly, cells (3,000/well) were seeded in 96-well culture plates the day before treatment and then incubated with drugs for 72h. Colorimetric reagent was then added to each well. After a 3h incubation at 37°C, the absorbance at 490 nm was measured, and IC₅₀ was determined by GraphPad Prism. Where indicated, assays were performed in the presence of 1 μg/mL of J591 or 10 μM of ZJ24 (Bachem Inc., Torrence, CA) to assess the effect on the activity of PSMA ADC.

Generation of docetaxel-refractory xenograft tumors  Male athymic nude mice (6 to 8 weeks old, Charles River Laboratories, Wilmington, MA) were each implanted with five
million C4-2 cells in Matrigel (Becton Dickinson, Franklin Lakes, NJ) by subcutaneous injection into the right flank. Fourteen days later, animals with tumor volumes between 100 mm³ and 200 mm³ were randomized to receive 2 mg/kg docetaxel (n=50) or vehicle (n=10) by weekly injection via the tail vein. This dose represents the maximum tolerated dose in this model (data not shown). Docetaxel was administered in PBS containing 0.6% ethanol and 1.5% polysorbate 80. Tumor volume, body weight, physical appearance and survival were recorded twice weekly. Tumor volume was calculated as (length) x (width²)/2.

**Treatment of docetaxel-refractory xenograft tumors** When the tumor volume of a docetaxel-treated animal exceeded 400 mm³, the animal was re-randomized 1:1 to receive weekly IV treatment with either 6 mg/kg PSMA ADC or 2 mg/kg docetaxel. Animals were monitored twice weekly and euthanized when in distress or when tumor volume exceeded 2,000 mm³. The study was terminated 182 days after tumors were implanted. The study was conducted in accordance with Institutional Animal Care and Use Committee guidelines.

**Statistical analyses** Inter-group differences in tumor volume and body weight were evaluated for significance via *t*-tests. Differences in survival and categorical variables were assessed using log-rank tests and Fisher’s exact tests, respectively. Results from two-tailed tests are reported. Statistical analyses were performed using GraphPad Prism.

**Results**

**PSMA expression in prostate cancer cells**
PSMA expression was first examined by Western blotting. PSMA was highest in MDA PCa2b cells followed by LNCaP cells, C4-2 cells, and CWR22rv1 cells (Figure 2A). PC3 cells and DU145 cells had no detectable expression of PSMA.

PSMA has carboxypeptidase activity, and its substrates include polyglutamated folates (35, 36). Folate hydrolase activity correlated well with PSMA expression observed by Western blotting (Figure 2B). Cells with higher expression of PSMA exhibited higher enzymatic activity. No enzymatic activity was observed in cells that were PSMA-negative by Western blotting.

PSMA expression was further characterized using ZJ24, a urea-based inhibitor of PSMA’s enzymatic activity (37). Binding results were fitted to a one-site binding model (Figure S1). Specific binding was observed only in PSMA-positive cells with K_D values between 35.6-46.5 nM (Table 1). The maximum binding capacities (Bmax) to PSMA-positive cells ranged from 26,600 to 319,000 molecules of ZJ24 per cell. The highest Bmax was observed in MDA PCA2b cells, which had the highest expression of PSMA by Western blotting. Next highest were LNCaP cells with Bmax of 251,900 molecules/cell, C4-2 cells with 204,900 molecules/cell and CWR22rv1 with 26,600 molecules/cell. PSMA is a symmetric dimer that contains two identical binding sites for ZJ24 (18, 38); therefore, expression of dimeric PSMA was estimated to range from 13,300 to 159,500 molecules/cell.

Cytotoxicity of PSMA ADC and control inhibitors towards prostate cancer cell lines

Human prostate cancer cell lines were examined for susceptibility to killing by PSMA ADC, free MMAE, unconjugated PSMA mAb and a control ADC of irrelevant specificity. The activity of PSMA ADC was highly dependent on PSMA expression,
with IC\textsubscript{50}~20 pM for cells with \(>10^5\) molecules/cell, IC\textsubscript{50}=804 pM for CWR22rv1 cells with \(10^4\) molecules/cell and IC\textsubscript{50}~30,000 pM for PSMA-negative cells (Figure 2C, Table 1). The control ADC showed weak activity (IC\textsubscript{50}~50,000 pM) that was independent of PSMA expression (Figure 2C, Table 1). Unconjugated PSMA mAb showed no cytotoxicity at concentrations ranging to 1 \(\mu\)M. MMAE had ~1,000 pM activity against each of the cell lines.

Docetaxel and paclitaxel are mitotic inhibitors whose mechanism of action resides in stabilizing microtubules in contrast with destabilizers such as MMAE (39). Docetaxel and paclitaxel were active against each of the prostate cancer cell lines, with IC\textsubscript{50} values of 1.2-7.5 nM and 5.9-46 nM, respectively (Figure 2C, Table 1). The taxols were modestly more effective against MDAPCa2b, LNCaP and C4-2 relative to PC3 and DU145.

The \textit{in vitro} potency of PSMA ADC was unaffected by the presence of 10 \(\mu\)M ZJ24, with respective IC\textsubscript{50} values of 22±14 pM and 29±18 pM observed in the presence and absence of ZJ24 (Figure 2D). In the presence of 1 \(\mu\)g/mL of anti-PSMA mAb J591, the potency of PSMA ADC was reduced approximately three-fold (IC\textsubscript{50}=106±32 pM).

**Cytotoxicity of PSMA ADC to PC3 cells transfected with wild-type or mutant PSMA**

To further investigate the determinants of PSMA ADC’s activity, PC3 cells were engineered to express wild-type PSMA (PC3wtPSMA), glycosylation mutant PSMA (PC3N638A), internalization mutant PSMA (PC3L5A) or vector control (PC3PIRESpuro). Transfectants were examined for PSMA expression, folate hydrolase activity, and for \(^3\)H-ZJ24 binding. Similar levels of wild-type and mutant forms of
PSMA ADC were detected by Western blotting (Figure 3A). Cells that expressed wild-type or internalization mutant PSMA exhibited folate hydrolase activity (Figure 3B) and specific binding of $^3$H-ZJ24 (Figure S2 and Table 1), whereas cells that expressed glycosylation mutant PSMA and vector-control cells did not exhibit either property.

PSMA ADC potently killed PC3wtPSMA cells ($IC_{50}=22$ pM) but not PC3PIRESpuro cells ($IC_{50}>30,000$ pM). PSMA ADC demonstrated minimal activity towards cells that expressed either the internalization mutant ($IC_{50}=8,170$ pM) or the glycosylation mutant ($IC_{50}=12,400$ pM). The glycosylation mutant adopts a non-native conformation (33, 34) and does not efficiently bind PSMA ADC (data not shown). The control ADC and PSMA mAb showed weak activity ($IC_{50}\geq30,000$ pM) and unmeasurable activity ($IC_{50}>1\mu$M), respectively, against each of the transfectants. MMAE killed each of the transfected cells with $IC_{50}$ of 594 to 775 pM (Figure 3C and Table 1).

**Generation of docetaxel-refractory xenograft tumors**

A total of 60 animals were included in the xenograft study (Figure 4A). At 14 days following tumor implantation, animals were randomized to receive docetaxel ($n=50$) or matched vehicle (Group 1, $n=10$). Of the 50 animals assigned to receive docetaxel, 14 animals had tumors that remained $<400$ mm$^3$, and these animals continued to receive docetaxel for the duration of the study (Group #2). Tumors grew to $>400$ mm$^3$ in the remaining 36 docetaxel-treated animals, and they were re-randomized 1:1 to receive PSMA ADC (Group #3) or to continue docetaxel treatment (Group #4). Because the rate of tumor growth varied in individual mice treated with docetaxel, mice were randomized continuously into Group #3 or Group #4 between days 25 and 119 post-implantation.
The mean tumor volume was 138 mm$^3$ in both the docetaxel and vehicle groups prior to initiation of therapy. First-line docetaxel therapy significantly inhibited tumor growth (Figure 4B). Relative to vehicle-treated animals, docetaxel-treated animals had significantly lower mean tumor volumes ($P \leq 0.03$) at days 21, 25 and 28, as well as a significantly lower rate of progressive tumors $>400$ mm$^3$ ($P=0.0014$). Animals in the vehicle group had a median survival of 70 days (range 42 to 168 days). Median survival in the docetaxel group could not be determined due to re-randomization of progressors. However, 14 docetaxel-treated animals (28%) did not develop tumors $>400$ mm$^3$ at any time during the study. Of these 14 animals, three died of unknown causes, and 11 survived until the end of the study.

**Treatment of docetaxel-refractory xenograft tumors**

Docetaxel-refractory tumors were defined as those that grew to $>400$ mm$^3$ in the presence of weekly IV therapy with 2 mg/kg docetaxel. Of the 50 animals that received first-line docetaxel therapy, 36 (72%) developed docetaxel-refractory tumors and were re-randomized 1:1 to receive PSMA ADC or continued treatment with docetaxel (Figure 4A). Mean tumor volumes were 515±103 mm$^3$ (range: 410 to 727 mm$^3$) and 495±80 mm$^3$ (range: 401 to 650 mm$^3$) for animals re-randomized to receive PSMA ADC (Group #3) or docetaxel (Group #4), respectively. The median time of re-randomization was 49 days post-implantation for animals in both Group #3 (range 25 to 119 days) and Group #4 (range 25 to 98 days).

Figure 5 illustrates the change in tumor volumes of the 36 animals that were re-randomized to receive PSMA ADC or continued docetaxel. Day 0 represents the time of
re-randomization to PSMA ADC or docetaxel. Data prior to day 0 depict changes in tumor volumes while the animals received first-line docetaxel therapy.

Tumor volumes decreased in all 18 animals treated with PSMA ADC (Figure 5A). Rapid regressions were observed even for tumors that were $>700$ mm$^3$. Although the kinetics of tumor regression varied somewhat (Figure 5A), 17 of 18 mice (94%) in the PSMA ADC group (Group #3) had $<100$ mm$^3$ tumors at the end of the study. Tumor volume was 166 mm$^3$ in the remaining Group #3 animal. In animals that continued to receive docetaxel, tumors progressed to $>2,000$ mm$^3$ in all but two animals whose tumor volumes were 275 mm$^3$ and 1,403 mm$^3$ at the end of the study (Figure 5B). The difference in the rate of tumor progression between Group #3 and Group #4 was highly statistically significant ($P<0.0001$).

Mean tumor volume was significantly lower in the PSMA ADC group relative to the docetaxel group (Figure 5C; $P\leq0.0005$) at all timepoints from 3 to 59 days following re-randomization. Thereafter, few animals remained in the docetaxel group (n$\leq$6). From an initial value of 515 mm$^3$ prior to initiation of PSMA ADC therapy, mean tumor volumes decreased to $<100$ mm$^3$ within 38 days of treatment and remained $<100$ mm$^3$ for the duration of the study.

Treatment with PSMA ADC significantly improved survival ($P<0.0001$, Figure 5D). At the end of the study, all 18 animals in the PSMA ADC group (Group #3) were alive. The median duration of treatment with PSMA ADC was 129 days (range: 87 to 157 days). In contrast, median survival for Group #4 animals was 45 days following re-randomization, and only 2 of 18 animals survived through the end of the study.
PSMA ADC was not associated with any overt toxicity. Physical appearance and activity were unaffected. Mean body weight increased for animals in the PSMA ADC group and decreased for animals in the docetaxel group. The difference in mean body weights of the two groups was significant ($P \leq 0.0001$) starting 17 days after re-randomization. The PSMA ADC group had a 20.6% increase in mean body weight 59 days after re-randomization, while the docetaxel group had a 14.5% decrease ($P < 0.0001$). Mean body weight remained stable or increased slightly in the PSMA ADC group thereafter; however, there were too few animals remaining in the docetaxel group for meaningful statistical comparisons.

**Discussion**

We report *in vitro* and *in vivo* studies of the antitumor activity of PSMA ADC, an anti-PSMA mAb conjugated to vcMMAE. Potent cytotoxicity ($IC_{50} \leq 20$ pM) was observed for androgen-dependent and -independent cells with $\geq 10^5$ molecules of PSMA per cell, and $10^4$ molecules per cell served as a threshold level of PSMA expression for selective cytotoxic activity. We also describe the *in vivo* efficacy of this agent in an *in situ* model of taxane-refractory human prostate cancer. PSMA ADC was highly active against tumors that had progressed following an initial course of docetaxel therapy, and even large (>700 mm$^3$) tumors showed rapid regressions. Our findings establish pharmacological determinants of activity of a novel antibody-drug conjugate. In addition, our *in vivo* model may prove useful for studying second-line therapy with other agents in other tumor settings.
PSMA ADC showed potent and selective activity against PSMA-positive prostate cancer cells. Cells insensitive to PSMA ADC were the PSMA-negative PC3 and DU145 lines, which are poorly differentiated lines. PC3 cells became susceptible to killing by PSMA ADC ($IC_{50}=22$ pM) following transfection with PSMA. In contrast to PSMA ADC, a control ADC of irrelevant specificity, free MMAE, and other microtubule inhibitors exhibited less potent \textit{in vitro} activity that was independent of PSMA expression. Unconjugated PSMA mAb did not exhibit intrinsic cytotoxic activity in this study, consistent with prior reports (31, 40, 41); however, our study did not examine the potential for cytotoxic effects mediated by mAb Fc effector functions.

The activity of PSMA ADC was dependent on internalization and proper folding of PSMA, as reduced cytotoxicity was observed against PC3 cells transfected with internalization or N-glycosylation mutant forms of PSMA (33, 42). These findings are consistent with PSMA ADC’s primary mechanism of action of delivering a potent cytotoxin to the interior of cells that express and internalize native PSMA.

Internalization of PSMA is mediated by a MWNLL motif in its cytoplasmic tail (26). Mutation of this motif did not affect PSMA’s enzymatic activity, and the cytotoxicity of PSMA ADC was unaffected by the PSMA inhibitor ZJ24, indicating that internalization and enzymatic activity are unrelated processes. In contrast, mAb J591, which reduces cell-surface levels of PSMA through internalization, reduced the potency of PSMA ADC ~3-fold, but complete cell killing was achieved at higher concentrations of PSMA ADC.

Besides being a target for therapy, PSMA has been used to image tumors. Unfortunately, the original imaging agent $^{111}$In-capromab pendetide binds an intracellular
site on PSMA and has limited utility in imaging bone metastases. Second-generation mAbs to the external domain of PSMA have been found to image bone metastases with nearly 100% accuracy (19). Because imaging would reflect not only site but potentially degree of PSMA expression, imaging tumors before and after therapy could potentially provide a means to assess tumor responses at individual sites. Unlike mAbs, low molecular weight imaging agents do not induce internalization of PSMA and therefore have the potential advantage of not interfering with the antitumor activity of ADCs.

Once prostate cancer becomes refractory to androgen deprivation, chemotherapy with taxanes is indicated; however, the side effects of therapy are appreciable relative to the limited survival benefit. PSMA expression increases following androgen-deprivation (22, 43). When tested against androgen-independent C4-2 cells in our study, PSMA ADC was >30-fold more potent than docetaxel and paclitaxel. PSMA ADC had minimal activity against PSMA-negative cells, in keeping with the desired reduction in off-target toxicity. PSMA ADC demonstrated selective potency both in androgen-dependent (MDA PCa2b and LNCaP) and androgen-independent (C4-2 and CWR22rv1) PSMA-expressing cell lines, all of which were derived from metastatic disease. The activity of PSMA ADC correlated with PSMA expression in these cells.

Our in vivo model reproduces several important features of docetaxel-refractory prostate cancer, including androgen independence, disease progression following an initial response to docetaxel, inter-subject variation in rates of progression, and the presence of tumor-stroma interactions. Docetaxel significantly reduced tumor growth ($P=0.0014$) and prevented disease progression in a substantial fraction of treated animals. By these measures, the efficacy seen in this preclinical model is comparable to
docetaxel’s clinical efficacy (44). In addition, as seen clinically, most docetaxel-treated tumors eventually progressed and became insensitive to further treatment with docetaxel.

Treatment of docetaxel-refractory tumors with PSMA ADC led to significant improvements in mean tumor volume ($P<0.0005$) and survival ($P<0.0001$) relative to continued docetaxel treatment. In addition, mean body weight increased significantly ($P<0.0001$) in the PSMA ADC group relative to the continued docetaxel group. The 60-animal study, therefore, was sufficiently powered for these endpoints. This approach could potentially be adapted to preclinically assess second-line chemotherapies in other settings.

The efficacy for PSMA ADC observed here is consistent with our prior report, which examined treatment of intramuscular C4-2 tumors (31). In both studies, all tumors responded to treatment with 6 mg/kg PSMA ADC. One important difference between the two studies is the duration of treatment. Our prior study employed a q4dx6 regimen, and tumors rebounded following cessation of treatment in three of five animals. The present study examined continuous treatment, which resulted in continued tumor suppression. PSMA ADC showed consistent, high-level activity against tumors up to 700 mm$^3$ in size in the present study as well as against smaller, disseminated tumors in our previous study. These findings are translationally relevant for prostate cancer, where many patients have micrometastatic tumors that are poorly imaged by conventional radiological methods. Neither unconjugated PSMA mAb nor control ADC demonstrated measurable efficacy against xenografted C4.2 tumors in our prior study (31), indicating that the primary mechanism of antitumor activity in this setting is mAb-mediated delivery
of MMAE to PSMA-expressing cells and not nonspecific release of free MMAE or other factors.

The preclinical antitumor activity of PSMA ADC compares favorably with that reported for MLN2704, an ADC comprising humanized J591 linked to drug maytansinoid 1 (40). Against LNCaP cells, the in vitro IC$_{50}$ for PSMA ADC (39 pM) is approximately 50-fold less than that reported for MLN2704. In addition, weekly treatment of subcutaneous CWR22 xenografts with 12.9 mg/kg MLN2704 resulted in tumor growth delay but limited tumor regression (40), whereas potent tumor regressions were seen here with 6 mg/kg weekly doses of PSMA ADC. Experimental differences between the two studies, however, preclude direct comparison of results.

Several in vitro mechanisms of docetaxel resistance have been identified in studies of prostate cancer cells. The mechanisms include tubulin mutations, redistribution of tubulin isoforms, down regulation of thrombospondin-1, loss of PTEN activity, and induction of Stat1 and clusterin (5-8, 45). Less is known regarding clinical resistance. In particular, it remains uncertain whether ATP-binding cassette (ABC) transport proteins play a key role (2). Rather, multifactorial mechanisms have been implicated in clinical resistance to taxanes (2, 3).

The sharp contrast in tumor responses to PSMA ADC or continued docetaxel therapy provides insights into the potential docetaxel resistance pathways operative in the present study. Resistance to docetaxel is unlikely to reflect broad upregulation of ABC transporters since both taxanes and MMAE are transported by members of this protein family (46-48); however, we cannot exclude the possibility of selective upregulation of a transporter that confers resistance to docetaxel and not MMAE. Similarly, increased
interstitial fluid pressure is unlikely to be a primary cause of resistance, since this mechanism would affect macromolecules to a greater extent than small molecules (49). Docetaxel-refractory tumors maintained PSMA expression at levels sufficient for ADC therapy in this study; this finding is consistent with in vitro studies demonstrating that short-term exposure to docetaxel does not affect PSMA expression (50).

This study establishes determinants of PSMA ADC’s antitumor activity using a series of primary and engineered prostate cancer cell lines. Selective antitumor activity was observed only in PSMA-expressing cells with more than $10^4$ PSMA molecules per cell. Internalization and glycosylation-dependent folding of PSMA were important for the in vitro potency of PSMA ADC. In addition, PSMA ADC was broadly and potently active against human prostate tumors that had become refractory to docetaxel treatment in vivo. Our findings are relevant to the ongoing clinical investigation of this targeted agent in advanced prostate cancer.
ACKNOWLEDGMENTS

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# Tables with Descriptive Titles

## Table 1. PSMA expression and cytotoxicity data.

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<tr>
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<th>ZJ24 Binding</th>
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<td>LNCaP</td>
<td>Androgen dependent</td>
<td>251,900 ± 9,300</td>
<td>39.4 ± 2.9</td>
<td>125,950</td>
<td>0.019 ± 0.012</td>
<td>0.502 ± 0.093</td>
<td>78.5 ± 6.6</td>
<td>1.55 ± 0.18</td>
</tr>
<tr>
<td>C4-2</td>
<td>Androgen independent</td>
<td>204,900 ± 4,600</td>
<td>35.5 ± 0.8</td>
<td>102,450</td>
<td>0.011 ± 0.014</td>
<td>0.298 ± 0.075</td>
<td>57.8 ± 1.9</td>
<td>1.75 ± 0.10</td>
</tr>
<tr>
<td>CWR22rv1</td>
<td>Androgen independent</td>
<td>26,500 ± 500</td>
<td>35.6 ± 3.9</td>
<td>13,250</td>
<td>0.804 ± 0.167</td>
<td>1.44 ± 0.19</td>
<td>98.8 ± 5.4</td>
<td>3.55 ± 1.11</td>
</tr>
<tr>
<td>PC3</td>
<td>Androgen independent</td>
<td>no specific binding</td>
<td>N/A</td>
<td>N/A</td>
<td>83.4 ± 6.3</td>
<td>0.970 ± 0.174</td>
<td>96.2 ± 2.3</td>
<td>6.98 ± 0.89</td>
</tr>
<tr>
<td>DU145</td>
<td>Androgen independent</td>
<td>no specific binding</td>
<td>N/A</td>
<td>N/A</td>
<td>99.1 ± 5.4</td>
<td>0.911 ± 0.120</td>
<td>156.0 ± 5.4</td>
<td>7.47 ± 0.48</td>
</tr>
<tr>
<td>PC3PIRESpuro</td>
<td>PC3 transfection control</td>
<td>no specific binding</td>
<td>N/A</td>
<td>N/A</td>
<td>31.8 ± 3.7</td>
<td>0.775 ± 0.138</td>
<td>29.9 ± 3.7</td>
<td>ND</td>
</tr>
<tr>
<td>PC3wtPSMA</td>
<td>PC3 transfected with wild-type PSMA</td>
<td>75,800 ± 1,100</td>
<td>8.20 ± 1.46</td>
<td>37,900</td>
<td>0.022 ± 0.011</td>
<td>0.667 ± 0.150</td>
<td>38.5 ± 3.8</td>
<td>ND</td>
</tr>
<tr>
<td>PC3L5A</td>
<td>PC3 transfected with internalization mutant PSMA</td>
<td>20,700 ± 400</td>
<td>5.06 ± 2.63</td>
<td>10,350</td>
<td>8.17 ± 2.7</td>
<td>0.737 ± 0.175</td>
<td>32.4 ± 2.7</td>
<td>ND</td>
</tr>
<tr>
<td>PC3N638A</td>
<td>PC3 transfected with glycosylation mutant PSMA</td>
<td>no specific binding</td>
<td>N/A</td>
<td>N/A</td>
<td>12.4 ± 2.2</td>
<td>0.594 ± 0.110</td>
<td>48.2 ± 2.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data represent the mean±SD of triplicate determinations. Bmax = molecules of ZJ24 per cell. N/A = not applicable. ND = not done. PSMA mAb did not exhibit measurable cytotoxicity at concentrations ranging up to 1,000 nM.

*Expression levels of dimeric PSMA are estimated to be one-half of the Bmax observed for ZJ24 because PSMA is a symmetric dimer that contains two identical binding sites for ZJ24.
FIGURE LEGENDS

Figure 1. Structures of PSMA ADC and docetaxel.

Figure 2. Cytotoxicity of PSMA ADC in vitro. (A) Western blot analysis of PSMA in lysates from prostate cancer cells. PSMA was detected by J591. (B) Folate hydrolase assay. Cell membrane (2 µg) was incubated with 5 nmol of MTXGlu₂ for 1h. MTX formed was determined by HPLC. Data represent the mean±SD of triplicate determinations. (C) In vitro cytotoxicity. Cells were treated for 72h with varying concentrations of inhibitor and assessed for viability. (D) Effect of ZJ24 or J591 on the cytotoxicity of PSMA ADC. C4-2 cells were treated for 72h with varying concentrations of PSMA ADC in the presence or absence of 10 µM ZJ24 or 1 µg/mL J591 antibody.

Figure 3. Cytotoxicity of PSMA ADC to transfected PC3 cells. (A) Western blot analysis of PSMA in cell lysates. PSMA was detected by GCP.04. (B) Folate hydrolase assay of cell membrane. Cell membrane (2 µg) was incubated with 5 nmol of MTXGlu₂ for 1h. MTX formed was determined by HPLC. Data represent the mean±SD of triplicate determinations. (C) In vitro cytotoxicity. PC3 transfectants were treated for 72h with inhibitor and then assessed for viability. Data are presented for PC3 cells transfected with empty vector (PC3PIRESpuro), wild-type PSMA (PC3wtPSMA), internalization mutant PSMA (PC3L5A) or glycosylation mutant PSMA (PC3N638A).

Figure 4. Disposition of study animals and efficacy of first-line therapy with docetaxel. (A) Disposition of animals amongst treatment groups. Fourteen days after tumor implantation, animals with 100-200 mm³ tumors were randomized to receive
docetaxel (2 mg/kg IV weekly, n=50) or matched vehicle (Group #1, n=10). When tumor volume in the docetaxel-treated group exceeded 400 mm$^3$, the animal was re-randomized 1:1 to receive PSMA ADC (6 mg/kg IV weekly, Group #3) or continued docetaxel treatment (2 mg/kg IV weekly, Group #4). Mice whose tumors responded durably to docetaxel ($\leq$400 mm$^3$) continued to receive docetaxel at 2 mg/kg IV weekly (Group #2). The experiment was terminated 182 days following tumor implantation. (B) Efficacy of first-line docetaxel therapy. Mean tumor volumes (± SEM) of docetaxel- or vehicle-treated animals are plotted up to the time the first docetaxel-treated animals were re-randomized due to tumor progression.

**Figure 5. Efficacy of PSMA ADC against docetaxel-refractory tumors.** Docetaxel-treated mice with tumors of >400 mm$^3$ were re-randomized 1:1 to receive weekly IV treatment with 6 mg/kg PSMA ADC (n=18) or 2 mg/kg docetaxel (n=18). Tumor volumes are shown for individual animals treated with PSMA ADC (A) or docetaxel (B). (C) Mean tumor volumes (±SEM). Mean tumor volume was significantly lower ($P\leq0.0005$) in the PSMA ADC treatment group relative to the docetaxel group. (D) Kaplan Meier survival curves. At the end of the study, 18 of 18 animals in the PSMA ADC group and 2 of 18 animals in the docetaxel group were alive ($P<0.0001$). Median survival for the docetaxel group was 45 days after re-randomization.
Figure 1, Wang et al. MCT-11-0191
Figure 2, Wang et al. *MCT-11-0191*
Figure 3, Wang et al. MCT-11-0191
A

60 with tumors 100-200 mm³ were randomized

50 received docetaxel 2 mg/kg IV weekly

10 received vehicle IV weekly (Group #1)

14 had non-progressive tumors (≤400 mm³) and continued to receive docetaxel (Group #2)

36 had progressive tumors (>400 mm³) and were re-randomized

18 received PSMA ADC 6 mg/kg IV weekly (Group #3)

18 received docetaxel 2 mg/kg IV weekly (Group #4)

B

![Graph showing tumor volume over days post tumor implantation]

- Vehicle (n = 10)
- Docetaxel, 2 mg/kg (n = 50)

Figure 4, Wang et al. MCT-11-0191
Figure 5, Wang et al. MCT-11-0191
Molecular Cancer Therapeutics

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Xinning Wang, Dangshe Ma, William C. Olson, et al.

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