Potent Antitumor Activity of an Auristatin-Conjugated, Fully Human Monoclonal Antibody to Prostate-Specific Membrane Antigen

Dangshe Ma,1 Christine E. Hopf,1 Andrew D. Malewicz,1 Gerald P. Donovan,1 Peter D. Senter,2 William F. Goeckeler,1 Paul J. Maddon,1 and William C. Olson1

Abstract Prostate-specific membrane antigen (PSMA) is the prototypic cell-surface marker of prostate cancer and provides an attractive target for monoclonal antibody (mAb) targeted therapies. In this study, a novel antibody-drug conjugate (ADC) was generated by linking a fully human PSMA mAb to monomethylauristatin E (MMAE), a potent inhibitor of tubulin polymerization. The PSMA ADC was evaluated for antitumor activity in vitro and in a mouse xenograft model of androgen-independent human prostate cancer. The PSMA ADC eliminated PSMA-expressing cells with picomolar potency and >700-fold selectivity in culture. When used to treat mice with established human C4-2 tumors, the PSMA ADC significantly improved median survival 9-fold relative to vehicle or isotype-matched ADC (P = 0.0018) without toxicity. Treatment effects were also manifest as significant (P = 0.0068) reduction in serum levels of prostate-specific antigen (PSA). Importantly, 40% of treated animals had no detectable tumor or measurable PSA at day 500 and could be considered cured. The findings support development of PSMA antibody-auristatin conjugates for therapy of prostate cancer.

Prostate cancer is the most common malignancy and the second leading cause of cancer death in men in the United States (1). Localized prostate cancer typically is treated with surgery or radiation, and recurrent disease can be controlled temporarily with androgen ablation (2). However, almost all prostate carcinomas eventually become hormone refractory and then rapidly progress (3). Hormone-refractory or androgen-independent prostate cancer has proven to be largely resistant to conventional chemotherapy. With the exception of palliative care, the only approved chemotherapy is docetaxel in combination with prednisone, which offers a modest (2.4 months) survival benefit (4, 5). New molecularly targeted therapies are urgently needed.

Monoclonal antibody (mAb) therapies have shown considerable use in the treatment of non-Hodgkin's lymphoma (6), B-cell chronic lymphocytic leukemia (7), colorectal carcinoma (8, 9), and breast cancer (10). In addition, mAbs have proven effective as vehicles to deliver therapeutic drugs or radionuclides in acute myeloid leukemia (11) and non-Hodgkin’s lymphoma (12, 13). Antibody-drug conjugates (ADC) combine the molecular targeting of mAbs with the chemotherapeutic properties of potent cytotoxins and hold great promise as molecularly targeted therapies of cancer.

Prostate-specific membrane antigen (PSMA) is a very well characterized cell surface marker of prostate cancer (14–17). PSMA is a homodimeric type II integral membrane glycoprotein whose expression is largely restricted to the prostate and is highly up-regulated in prostate carcinomas (17, 18). Its expression increases with disease progression, becoming highest in metastatic, hormone-refractory disease (19). In addition, PSMA is abundantly expressed on the neovascularature of most other solid tumors but not on normal vasculature (20–24). Importantly, PSMA is rapidly and constitutively internalized, facilitating delivery of ADCs into the cell interior (25, 26). These properties render PSMA an attractive target for ADC therapy of prostate and other cancers.

We previously described the preparation and characterization of fully human mAbs to conformational and dimer-specific epitopes within the extracellular domain of PSMA (17). Fully human mAbs are indistinguishable from naturally occurring human antibodies and thus offer the potential for repeat administration without the development of a neutralizing immune response. The auristatins are synthetic antimitotic agents related to the natural product dolastatin 10 (27) and are ~200 times more potent in vitro than conventional chemotherapeutic agents (28). A dipeptide linkage cleavable by lysosomal proteases has recently been applied to link auristatin-based drugs to mAbs (29–33). In this report, a novel ADC was generated by conjugating a PSMA mAb to monomethylauristatin E (MMAE) through a valine-citulline (Val-Cit) linkage designed to maintain serum stability while maximizing intracellular drug release by human cathepsin B.

Note: PSMA Development Co., LLC is a joint venture between Cytogen Corp. (Princeton) and Progenics Pharmaceuticals, Inc.

Requests for reprints: Dangshe Ma, Progenics Pharmaceutiques, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591. Phone: 914-789-2800; Fax: 914-789-2807; E-mail: dma@progenics.com.

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Cancer Therapy: Preclinical
We report that the PSMA ADC potently and specifically eliminated human prostate cancer cells in in vitro and in vivo models of androgen-independent prostate cancer.

Materials and Methods

Cell lines and antibodies. LNCaP, PC-3, and 3T3 were obtained from the American Type Culture Collection (Rockville, MD). C4-2 cell line, a subcell line from LNCaP, was provided by Dr. Warren D.W. Heston (The Cleveland Clinic Foundation, Cleveland, OH). A 3T3-PSMA cell line was a generous gift of Dr. Michel Sadelain (Memorial Sloan-Kettering Cancer Center, New York, NY). LNCaP, C4-2, and PC-3 were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD), and 3T3 and 3T3-PSMA were cultured in DMEM (Life Technologies). Culture media were supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), L-glutamine, penicillin, and streptomycin (Life Technologies). C4-2, LNCaP, and 3T3-PSMA cells were determined to express PSMA at levels of \( \geq 2 \times 10^5 \), \( \geq 6 \times 10^5 \), and \( >1 \times 10^6 \) copies per cell, respectively, according to published methods (34). C4-2 is an androgen-independent subclone of androgen-dependent LNCaP cells (35). PC-3 is a differentiated prostate cancer cell line that does not express PSMA. A fully human PSMA mAb (IgG1, \( \kappa \)) was raised in mice transgenic for the human immunoglobulin gene locus (XenoMice, Abgenix, Inc., Fremont, CA) following immunization with recombinant soluble PSMA and LNCaP cells as previously described (17). PSMA mAb binds a conformation-dependent epitope within the extracellular domain of PSMA.

PSMA internalization. mAbs were modified with bifunctional chelates of cyclohexyl-dieihylenetriamine pentaacetic acid (CHX-DTPA) generously provided by Dr. Martin Brechbiel (National Cancer Institute, Bethesda, MD) and labeled with \( ^{111} \text{In} \) (Perkin-Elmer, Boston, MA) as previously described (34, 36). \(^{111} \text{In}-\)labeled mAb was determined to be \( >90\% \) immunoreactive by incubating the radiolabeled PSMA mAb and LNCaP cells as previously described (34). For internalization analysis, \( ^{111} \text{In}-\)labeled mAb was incubated with \( 2 \times 10^5 \) C4-2 cells at \( 37^\circ C \) for 30 minutes. The mAb was eluted using low pH buffer (pH 2.4, glycine/NaCl) and counted separately from the cell pellet, and percent internalization was calculated as previously described (37).

Preparation of ADCs. The conjugation of mAbs with maleimido-caproyl-Val-Cit-monomethylauristatin E was done as described elsewhere (28). Briefly, PSMA mAb and isotype-control human IgG1 (Calbiochem, San Diego, CA) in PBS containing 50 mmol/L borate (pH 8) were treated with DTT (10 mmol/L final) at 37°C for 30 minutes. The mAbs were exchanged into PBS containing 1 mmol/L DTPA (Aldrich, Milwaukee, WI) by passage through a Sephadex G-25 column (Amersham Biosciences, Piscataway, NJ). The mAb solutions were chilled to 4°C and combined with the maleimido drug derivative in cold CHCl3. After 1 hour, the reactions were quenched with excess cysteine, and the conjugates were concentrated and exchanged into PBS buffer. The ADCs were determined to have \( >98\% \) monomeric mAb containing 3.0 to 3.5 drugs per mAb using published methods (28). The drug/antibody ratio was selected empirically based on findings for other auristatin ADCs (31).

Reactivity of ADCs with cell-surface PSMA. Binding of PSMA mAb and ADC to 3T3-PSMA cells. 3T3-PSMA cells were incubated with increasing concentrations of the PSMA mAb (■), PSMA ADC (○), or isotype-control ADC (○). Cells were then incubated with goat anti-human IgG-FITC, washed again, and examined by a flow cytometer. The mean fluorescence intensities are plotted as a function of mAb or ADC concentration.
IgG-FITC (Caltag Laboratories, Burlingame, CA). Isotype-control antibody and ADC were examined in parallel.

In vitro cytotoxicity assay. PMMA-positive cells (C4-2, LNCaP, or 3T3-PSMA) and PMMA-negative cells (PC-3 or 3T3) were added to 96-well microplates (Falcon, Franklin Lakes, NJ) at 2.5 × 10^4 per well and incubated overnight at 37°C and 5% CO_2. Plates were then read on a fluorescence plate reader using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Cell survival was compared in treated and untreated cultures, and the concentration of ADC required for 50% cell kill (IC_{50} value) was determined.

Results

Internalization of the PMMA mAb into human prostate cancer cells. Internalization was examined using ^111^In-labeled PMMA mAb and C4-2 cells. Total binding and percent internalization over time are illustrated in Fig. 1. Over half of the bound mAb was internalized within 2 hours (Fig. 1A). Total binding increased over time, presumably due to PMMA recycling (Fig. 1B). Thus, the PMMA mAb is readily internalized into PSMA-expressing cells.

In the first experiment, animals were randomized at day 17 and treated q4d × 3 with 0, 2, or 10 mg/kg PMMA ADC. In vehicle control group, tumors grew rapidly, and animals had a median survival of 32 days. In contrast, the groups treated with 2 and 10 mg/kg PMMA ADC had median survivals of 58 days (P = 0.0035) and 95 days (P = 0.0010), respectively (Table 1; Fig. 4A). The PMMA ADC significantly improved median survival up to 5-fold post-treatment in a dose-dependent fashion. There was no evidence of treatment-related toxicity.

Table 2. Median survival times of C4-2 tumor-bearing animals treated with PMMA ADC

<table>
<thead>
<tr>
<th>Test article</th>
<th>Dose (mg/kg)</th>
<th>Schedule</th>
<th>Median survival (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>NA</td>
<td>q4d × 3</td>
<td>32</td>
</tr>
<tr>
<td>PSMA ADC</td>
<td>2</td>
<td></td>
<td>58*</td>
</tr>
<tr>
<td>PSMA ADC</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>NA</td>
<td>q4d × 6</td>
<td>29</td>
</tr>
<tr>
<td>PSMA mAb</td>
<td>6</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>Control ADC</td>
<td>6</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>PSMA ADC</td>
<td>3</td>
<td></td>
<td>49*</td>
</tr>
<tr>
<td>PSMA ADC</td>
<td>6</td>
<td></td>
<td>148*</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.

*Significant (P < 0.005) improvement in survival over vehicle control group in a two-sided log-rank analysis.

**Reactivity of the PMMA ADC with PMMA-expressing cells.** Flow cytometry was used to compare the binding of PMMA mAb and ADC. The unmodified mAb and ADC showed comparable levels of binding to 3T3-PSMA over a broad range of dilutions (Fig. 2). Neither the maximal amount of binding nor the concentration required for half-maximal binding was appreciably affected by conjugation. No significant binding was observed for the isotype control ADC or antibody on 3T3-PSMA cells or for PMMA mAb or ADC on parental 3T3 cells (data not shown).

**In vivo potency and selectivity of the PMMA ADC.** PMMA and control ADCs were tested for cytotoxicity in vitro against human prostate cancer cells lines and 3T3-PSMA cells. Figure 3 illustrates dose-response curves for PMMA-positive C4-2 cells (Fig. 3A) and PMMA-negative PC-3 cells (Fig. 3B) in a representative experiment, and IC_{50} values for the various cell lines are listed in Table 1. The PMMA ADC potently eliminated all PMMA-positive cell lines examined at IC_{50} values of 65 to 210 pmol/L, whereas these concentrations had no effect on PMMA-negative cells. In contrast, nearly 1,000-fold higher concentrations were required for the control ADC, whose activity was independent of PMMA expression (Fig. 3; Table 1).

Efficacy of the PMMA ADC in xenograft models of androgen-independent prostate cancer. In vivo efficacy of the PMMA ADC was evaluated in a mouse model of androgen-independent human prostate cancer. Nude mice were engrafted with C4-2 cells i.m. in the left hind leg. Fourteen or 17 days later, serum PSA levels were measured and used to randomly assign animals to treatment groups. Animals were treated i.v. with the PMMA ADC, and animals were monitored for tumor burden, PSA levels and other variables for as long as 500 days.

In the first experiment, animals were randomized at day 17 and treated q4d × 3 with 0, 2, or 10 mg/kg PMMA ADC. In vehicle control group, tumors grew rapidly, and animals had a median survival of 32 days. In contrast, the groups treated with 2 and 10 mg/kg PMMA ADC had median survivals of 58 days (P = 0.0035) and 95 days (P = 0.0010), respectively (Table 2; Fig. 4A). The PMMA ADC significantly improved median survival up to 5-fold post-treatment in a dose-dependent fashion. There was no evidence of treatment-related toxicity.

Table 1. Summary of in vitro cytotoxicity (IC_{50} values in pmol/L)

<table>
<thead>
<tr>
<th></th>
<th>C4-2</th>
<th>LNCaP</th>
<th>3T3-PSMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMMA ADC</td>
<td>65 ± 19 (n = 3)</td>
<td>83 ± 21 (n = 2)</td>
<td>208 ± 37 (n = 3)</td>
</tr>
<tr>
<td>Control ADC</td>
<td>54,954 (n = 1)</td>
<td>72,444 (n = 1)</td>
<td>154,880 (n = 1)</td>
</tr>
<tr>
<td>Selectivity*</td>
<td>848</td>
<td>877</td>
<td>744</td>
</tr>
</tbody>
</table>

*Selectivity equals the ratio of IC_{50} values observed for the PMMA ADC and control ADC.
Serum PSA levels were measured over time by ELISA. Figure 4B depicts the mean PSA concentration in each group at study days 17, 23, and 30. Treatment at 10 mg/kg reduced PSA levels >10-fold from 8.8 ± 11.7 ng/mL at day 17 to 0.7 ± 0.9 ng/mL at day 30, whereas PSA levels in control group increased >60-fold over the same time period. An intermediate response was observed with 2 mg/kg PSMA ADC. The differences in PSA levels at day 30 were significant for both the 2 mg/kg (P = 0.0048) and 10 mg/kg (P = 0.0006) dose groups. Three of six animals in the 10 mg/kg group had undetectable PSA through day 52 of the study.

To extend these findings, a second PSMA ADC study that also included unmodified mAb and isotype-control ADC was conducted. After randomization at day 14 with a mean PSA level of 2.0 ± 1.1 ng/mL in each group (n = 5), animals were treated with a regimen of q4d × 6. Kaplan-Meier survival curves for each group are depicted in Fig. 5. Animals treated with vehicle control, 6 mg/kg unmodified PSMA mAb and 6 mg/kg control ADC had similar median survival times of 29, 31, and 31 days, respectively; in addition, these differences were not significant. However, median survival was extended to 49 and 148 days for animals treated with 3 and 6 mg/kg PSMA ADC, respectively (Table 2). Treatment with 6 mg/kg PSMA ADC group improved post-randomization survival 9-fold relative to the vehicle control group (P = 0.0018). At day 500, two of five animals had no evidence of tumor, had no measurable PSA, and were considered cured by treatment. As in the first study, treatment had a significant effect on PSA levels on day 29 (P = 0.0068 for 6 mg/kg PSMA ADC and vehicle groups). Moreover, in the 6 mg/kg PSMA ADC group, serum PSA decreased to undetectable levels post-treatment and remained undetectable through day 63 in four of five animals.

There was no overt toxicity associated with ADC therapy in either study. Physical appearance and activity were unaffected by treatment, and body weights of treated and vehicle-control animals were not significantly different at any time point (data not shown).

**Discussion**

This report describes initial preclinical evaluations of a novel ADC comprising a fully human PSMA mAb and an auristatin chemotherapeutic agent. In *in vitro* and *in vivo* models of human prostate cancer, the PSMA ADC showed potent and selective antitumor activity. These findings are consistent with the abundant expression and rapid internalization of PSMA in prostate cancer cells, and the results support further development of this strategy for prostate and other cancers.
The PSMA ADC showed picomolar potency against three PSMA-expressing cells in culture with nearly 1,000-fold selectivity compared with an isotype control ADC. This result shows the specificity of mAb binding and the stability of the drug linker. Comparable activity was observed using androgen-dependent and androgen-independent prostate cancer cell lines. The PSMA ADC was ~3-fold more active against prostate cancer cells that express PSMA endogenously compared with 3T3-PSMA transfectants. There was no obvious dependence of ADC activity over a >4-fold range of PSMA expression.

The selective cytotoxicity of PSMA ADC also reflects efficient internalization of the antigen. PSMA is rapidly internalized in the presence and absence of PSMA mAb (Fig. 1A) and other antibodies (25, 38). Rapid internalization in prostate and nonprostate cell lines has been mapped to the MWNLL sequence in the cytoplasmic domain of PSMA (39).

![Fig. 5. Kaplan-Meier survival curves of animals treated in xenograft study 2.](image)

The PSMA ADC showed therapeutic efficacy in a mouse xenograft model of androgen-independent prostate cancer. The C4-2 model was used because these tumors (a) grow aggressively, (b) are androgen independent and therefore mimic our intended patient population, and (c) secrete PSA that can be monitored as a surrogate marker of tumor progression. Each of the two regimens tested were effective in increasing median survival and decreasing serum PSA in a dose-dependent fashion. Remarkably, a 40% cure rate was observed in animals with established tumors following treatment with 6 mg/kg PSMA ADC (or ~100 μg MMAE equivalents/kg) administered via a q4d × 6 regimen. Cured animals had no detectable tumor or serum PSA for as long as 500 days after implantation. The dose of 6 mg/kg with a q4d × 6 regimen was comparable with or less intensive than some agents evaluated for prostate cancer therapy in preclinical models [12.9 mg/kg with q3d × 5 (26) or 5 mg/kg with q4d × 13 (29)]. Because no apparent toxicities were observed with this regimen, further improvements in antitumor activity may be attainable. Anti-tumor effects were observed at lower doses as well, and the findings indicate that this agent possesses an appropriate therapeutic window in this model.

In addition to being expressed on malignant epithelial cells in prostate cancer, PSMA also is abundantly expressed in association with most other solid tumors on the endothelial cells of the tumor neovasculature. The tumor endothelium constitutes a single-layer target that represents another attractive setting for PSMA ADC therapy.

Our findings show that mAb-auristatin conjugates have potent and selective antitumor activity in *in vitro* and *in vivo* models of androgen-independent human prostate cancer. This approach merits further development as a molecularly targeted therapy of prostate and other cancers.

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**References**

6. Dillow RO. Magic bullets at last! Finally: approval of PSMA ADC for prostate cancer therapy in preclinical models [12.9 mg/kg with q3d × 5 (26) or 5 mg/kg with q4d × 13 (29)]. Because no apparent toxicities were observed with this regimen, further improvements in antitumor activity may be attainable. Anti-tumor effects were observed at lower doses as well, and the findings indicate that this agent possesses an appropriate therapeutic window in this model.

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