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

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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 neovasculature 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-citrulline (Val-Cit) linkage designed to maintain serum stability while maximizing intracellular drug release by human cathepsin B.

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 ~ 2×10^5, 6×10^5, and >1×10^6 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 dedifferentiated prostate cancer cell line that does not express PSMA. A fully human PSMA mAb (IgG1, κ) was raised in
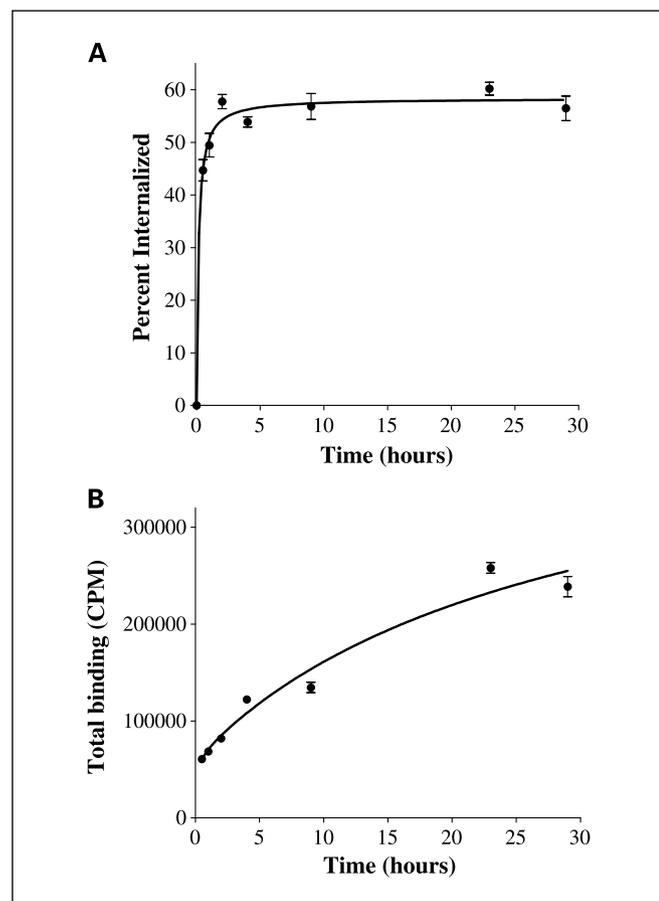

Fig. 2. Binding of PSMA mAb and ADC to 3T3-PSMA cells. 3T3-PSMA cells were incubated with increasing concentrations of the PSMA mAb (■), PSMA ADC (□), or isotype-control ADC (△). Cells were incubated on ice for 1 hour and washed to remove unbound mAb or ADC. The cells were then incubated with goat anti-human IgG-FITC, washed again, and examined by a flow cytometry. The mean fluorescence intensities are plotted as a function of mAb or ADC concentration.

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-diethylenetriamine pentaacetic acid (CHX-DTPA) generously provided by Dr. Martin Brechbiel (National Cancer Institute, Bethesda, MD) and labeled with [111]In (Perkin-Elmer, Boston, MA) as previously described (34, 36). ^{111}In-labeled mAb was determined to be >90% immunoreactive by incubating the radioconjugate with an excess of 3T3-PSMA cells and measuring the bound fraction according to published methods (34). For internalization analysis, ^{111}In-labeled mAb was incubated with 2×10^5 C4-2 cells at 37°C in 5% CO_2. At sequential time points, unbound mAb was removed by washing in PBS, and cell surface mAb was eluted using low pH buffer (pH 2.4, glycine/NaCl). The low pH eluate was counted separately from the cell pellet, and percent internalization was calculated as previously described (37).

Preparation of ADCs. The conjugation of mAbs with maleimidocaproyl-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 CH_3CN. 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 and parental 3T3 cells was analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA). Briefly, 2×10^5 3T3-PSMA (or 3T3) cells were incubated with different concentrations of mAb or ADC on ice for 1 hour. After washing, the presence of bound antibody was detected using goat anti-human

Fig. 1. Percent internalization and total binding of ^{111}In-labeled PSMA mAb on C4-2 cells. C4-2 cells were incubated with ^{111}In-labeled mAb at 37°C, 5% CO_2. At the designated times, cells were washed to remove unbound mAb, and surface-bound mAb was eluted using low pH buffer. The radioactivity (counts per minute or *CPM*) of the low pH eluate and cell pellet were counted separately using a gamma counter. Percent internalization (*A*) was calculated as the counts per minute cell pellet / (counts per minute cell pellet + counts per minute low pH eluate) × 100. Total binding (*B*) represents the counts per minute of the cell pellet plus the counts per minute of the low pH eluate.

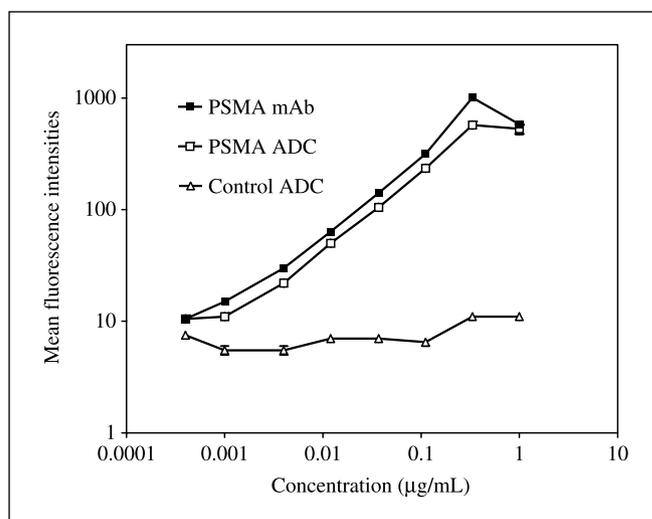IgG-FITC (Caltag Laboratories, Burlingame, CA). Isotype-control antibody and ADC were examined in parallel.

In vitro *cytotoxicity assay.* PSMA-positive cells (C4-2, LNCaP, or 3T3-PSMA) and PSMA-negative cells (PC-3 or 3T3) were added to 96-well microplates (Falcon, Franklin Lakes, NJ) at 2.5×10^3 per well and incubated overnight at $37°C$ and 5% CO_2. Cells were then incubated with serially diluted ADCs for 4 days. The cell culture medium was replaced with fresh medium containing 10% Alamar Blue (Biosource International, Camarillo, CA), and cells were incubated for 4 hours. 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.

Xenograft model of androgen-independent prostate cancer. All animal studies were carried out in accordance with Institutional Animal Care and Use Committee guidelines. Athymic male nude mice (National Cancer Institute, Frederick, MD) 6 to 8 weeks in age were implanted with an i.m. injection of 5×10^6 C4-2 cells mixed with Matrigel (Beckon Dickinson Labware, Bedford, MA) into the left hind leg as described (37). Before initiation of treatment, animals were randomized according to serum levels of prostate-specific antigen (PSA) as measured by ELISA (Medicorp, Montreal, Quebec, Canada). ADCs, mAb, and vehicle control were administered via tail vein injection. In the first series of experiments, mice were treated in groups of six with 2 or 10 mg/kg PSMA ADC or with vehicle control. Treatment was initiated 17 days after implantation and consisted of three injections at 4-day intervals (q4d × 3). This regimen was selected empirically based on that used in mouse studies of other auristatin ADCs (30). The second series of experiments examined dose levels of 0, 3, or 6 mg/kg. Treatment was initiated 14 days after implantation and consisted of six injections at 4-day intervals (q4d × 6). Animals were monitored for PSA level and tumor size. Survival rates were recorded throughout the studies. Treatment toxicity was assessed by body weight and physical appearance of the animals.

Statistical analyses. Treatment effects were examined for significance via t tests (for PSA levels) or log-rank tests (for animal survival) using two-tailed, paired analyses. Data were considered significant when $P < 0.05$.

Results

Internalization of the PSMA mAb into human prostate cancer cells. Internalization was examined using ^{111}In-labeled PSMA 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 PSMA recycling (Fig. 1B). Thus, the PSMA mAb is readily internalized into PSMA-expressing cells.

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

	C4-2	LNCaP	3T3-PSMA
PSMA ADC	65 ± 19 (n = 3)	83 ± 21 (n = 2)	208 ± 37 (n = 3)
Control ADC	54,954 (n = 1)	72,444 (n = 1)	154,880 (n = 1)
Selectivity*	848	877	744

*Selectivity equals the ratio of IC_{50} values observed for the PSMA ADC and control ADC.

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

Test article	Dose (mg/kg)	Schedule	Median survival (d)
Study 1			
Vehicle	NA	q4d × 3	32
PSMA ADC	2		58*
PSMA ADC	10		95*
Study 2			
Vehicle	NA	q4d × 6	29
PSMA mAb	6		31
Control ADC	6		31
PSMA ADC	3		49*
PSMA ADC	6		148*

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 PSMA ADC with PSMA-expressing cells. Flow cytometry was used to compare the binding of PSMA 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 PSMA mAb or ADC on parental 3T3 cells (data not shown).

In vitro *potency and selectivity of the PSMA ADC.* PSMA 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 PSMA-positive C4-2 cells (Fig. 3A) and PSMA-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 PSMA ADC potently eliminated all PSMA-positive cell lines examined at IC_{50} values of 65 to 210 pmol/L, whereas these concentrations had no effect on PSMA-negative cells. In contrast, nearly 1,000-fold higher concentrations were required for the control ADC, whose activity was independent of PSMA expression (Fig. 3; Table 1).

Efficacy of the PSMA ADC in xenograft models of androgen-independent prostate cancer. **In vivo** efficacy of the PSMA 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 PSMA 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 PSMA 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 PSMA ADC had median survivals of 58 days ($P = 0.0035$) and 95 days ($P = 0.0010$), respectively (Table 2; Fig. 4A). The PSMA ADC significantly improved median survival up to 5-fold post-treatment in a dose-dependent fashion. There was no evidence of treatment-related toxicity.
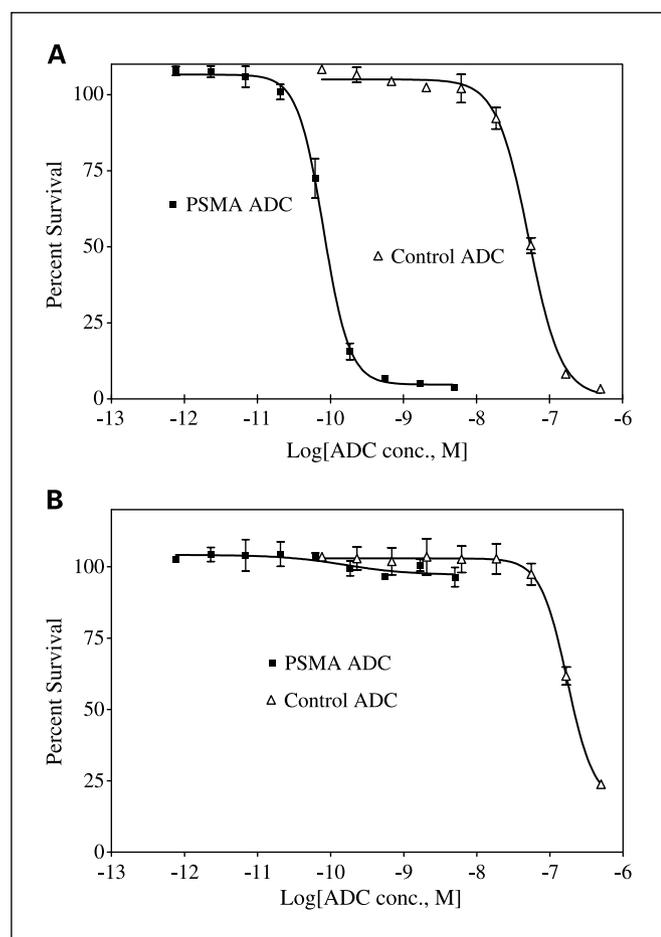
Fig. 3. *In vitro* cytotoxicity of the PSMA ADC and control ADC on PSMA-positive and PSMA-negative prostate cancer cell lines. PSMA-positive C4-2 cells (*A*) and PSMA-negative PC-3 cells (*B*) in 96-well microplates were exposed to ADCs at various concentrations. After 96 hours, cell survival in treated and untreated cultures was assayed using Alamar Blue.

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 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.
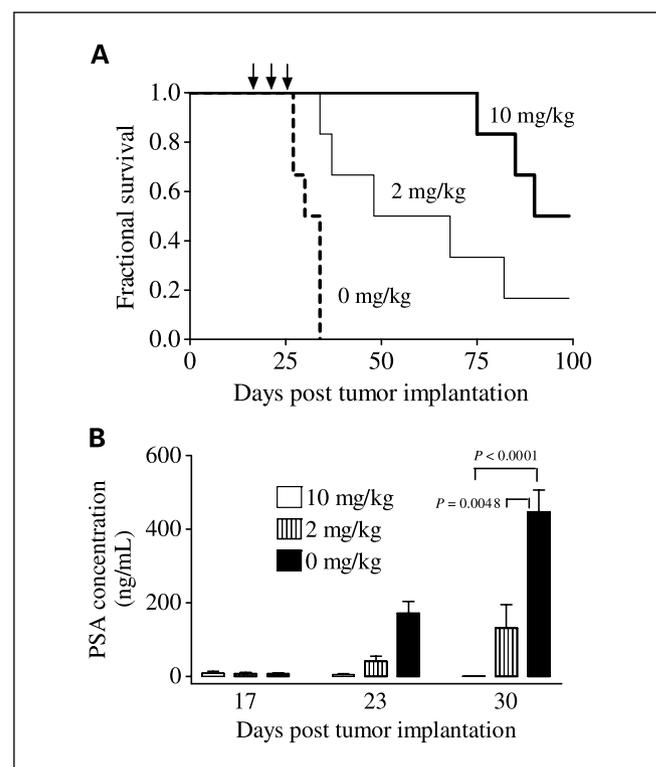

Fig. 4. Kaplan-Meier survival and serum PSA levels in xenograft study 1. Nude mice were implanted i.m. with C4-2 cells, randomly assigned to treatment groups (six mice per group) according to serum PSA on day 17 and then treated q4d × 3 with PSMA ADC or vehicle. *A*, survival of animals treated with 0 mg/kg (vehicle control, *dashed line*), 2 mg/kg (*thin solid line*), and 10 mg/kg PSMA ADC (*thick solid line*). *B*, mean PSA values over 30 days in mice treated with 0 mg/kg (*filled columns*), 2 mg/kg (*striped columns*), and 10 mg/kg (*open columns*) PSMA ADC. The day 30 data for the control group include day 27 evaluations for two mice that did not survive 30 days. The experiment was conducted as described in Materials and Methods.
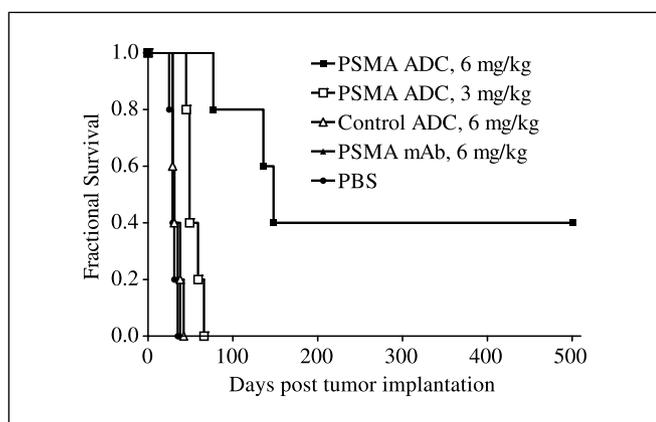
Fig. 5. Kaplan-Meier survival curves of animals treated in xenograft study 2. Nude mice were implanted i.m. with C4-2 cells, randomly assigned to treatment groups (five mice per group) according to serum PSA on day 14 and then treated q4d × 6 with PSMA ADC and controls. Mice were treated with 0 mg/kg (vehicle control, ●), 6 mg/kg unmodified PSMA mAb (▲), 6 mg/kg control ADC (△), 3 mg/kg PSMA ADC (□), and 6 mg/kg PSMA ADC (■). The experiment was conducted as described in Materials and Methods.

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).

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. Antitumor 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.

Acknowledgments

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