An Anti-PSMA Bivalent Immunotoxin Exhibits Specificity and Efficacy for Prostate Cancer Imaging and Therapy

Fayun Zhang, Liang Shan, Yuanyi Liu, David Neville, Jung-Hee Woo, Yue Chen, Alexandru Korotcov, Stephen Lin, Sophia Huang, Rajagopalan Sridhar, Wei Liang, and Paul C. Wang*

Prostate specific membrane antigen (PSMA) is overexpressed on prostate tumor cells and the neovascular endothelia various solid tumors. A bivalent immunotoxin generated by fusing a fold-back single-chain diabody derived from the Fv fragments of an anti-PSMA monoclonal antibody with a truncated diphtheria toxin (DT) containing the activity and translocation domains [A-dmDT390-scfDb(PSMA)] might be suitable for targeted therapy of tumors that overexpress PSMA. In this study, a PSMA-positive and a PSMA-negative prostate cancer cell lines were treated with immunotoxin A-dmDT390-scfDb(PSMA) in order to study the tumor targeting specificity and therapeutic potential of the immunotoxin. The cellular uptake and selective toxicity of the immunotoxin were evident in monolayer cultures of PSMA-positive LNCaP prostate cancer cells but not in cultures of PSMA-negative PC-3 prostate cancer cells. Cellular accumulation of A-dmDT390-scfDb(PSMA) increased with increasing incubation times and concentrations in LNCaP cells. The proportion of apoptotic LNCaP cells increased upon incubation with increasing doses of the fold-back immunotoxin. Optical imaging and MRI with the Alexa Fluor 680-labeled A-dmDT390-scfDb(PSMA) confirmed the specific targeting and therapeutic efficacy of this immunotoxin towards PSMA-positive LNCaP solid tumor xenografts in athymic nude mice.

1. Introduction

Prostate cancer is the most common solid tumor and one of the leading causes of cancer-related death among American men.[1] Radiotherapy and/or surgery with or without androgen deprivation are used for management of early stage, organ-confined prostate cancer. A subset of early stage cancer may progress to an aggressive metastatic disease, which does not respond to androgen deprivation. Chemotherapeutic approaches are used for treating metastatic prostate cancer. The development of androgen resistance and systemic off-target toxicities of conventional chemotherapeutic drugs such as docetaxel and mitoxantrone are major clinical challenges.[2, 3] There is a need for safe and effective therapies that are based on specific targeting of immunotoxins to tumors. Tumor cells often express high levels of surface receptors or other molecules that distinguish them from other cells. Ligands designed to bind to tumor-specific receptors can be conjugated to cytotoxic drugs or toxins and the resulting conjugates provide a tumor targeted drug delivery system for safe and effective therapy.[4] Further research along these lines may lead to molecularly targeted individualized therapy.

Prostate-specific membrane antigen (PSMA) is over-expressed on the surface of certain prostate cancer cells. It is noteworthy that PSMA expression is particularly pronounced when prostate cancer progresses to late stage and becomes androgen-independent and metastatic.[5] PSMA expression in certain prostate cancer cells is 1000-fold higher than in normal prostate tissue.[6] PSMA is also expressed on the neovascular endothelium of a wide variety of human solid tumors, but is not expressed in the blood vessels of normal tissue.[7] These findings have prompted the use of monoclonal antibody (mAb) of PSMA for sensitive and specific tumor imaging,[8] as well as targeted drug delivery for treating prostate cancer and other solid tumors.[9]

PSMA antibody or its fragments, such as single-chain antibody fragments (scFv), can deliver cytotoxic agents into PSMA-expressing cells.[10] scFv consists of the variable heavy chain (VH) and the variable light chain (VL) of an antibody connected by a flexible peptide linker and, due to its small size, exhibits...
better tumor penetration, improved tumor distribution, and faster blood clearance than a full antibody when it is used as a ligand for targeted drug delivery.\[^{11}\]

The truncated form of diphtheria toxin (DT390) constructs incorporated in the immunotoxin exhibits targeted cytotoxicity\[^{12,13}\] and bioactivity in vivo.\[^{14,15}\] The DT-based anti-CD3 bivalent immunotoxin, A-dmDT390-bisFv (UCHT1), has been shown to bind to its target and depletes T cells effectively.\[^{16}\] This immunotoxin has undergone preclinical studies\[^{17,18}\] and is currently in clinical trials for treating cutaneous T-cell lymphoma (clinical trial identifier NCT00611208). In general, bivalent immunotoxins containing two scFv units have higher affinity and efficacy towards the targeted cells than those containing one scFv.\[^{13,19}\] It has also been shown that the format of scFv bivalency strongly affects binding affinity and immunotoxin cytotoxicity, depending on the antibody Fv sequences. The anti-mono CD3 bivalent immunotoxin with a fold-back single-chain diabody, A-dmDT390-scfbDb (207), showed a 5- to 7-fold enhanced bioactivity over that containing two tandem units of scFv (biscFv).\[^{19}\] To effectively deliver DT390 and enhance the specificity of its targeting to PSMA-expressing cells, we constructed an anti-PSMA fold-back single-chain diabody (scfbDb).

**Figure 1.** The scheme of A-dmDT390-scfbDb comprising the A-dmDT390 moiety and the anti-PSMA scfbDb. (A): The diabody consists of two scFv fragments separated by optimized lengths of Gly-Ser linkers. (B): The immunotoxin comprises the A-dmDT390 moiety and the anti-PSMA scfbDb. The sequence from left to right is dmDT- VL-L1-VH-L2-VL-L1-VH. G4S are linkers, and VL and VH are the variable domains of light and heavy chains, respectively; A-dmDT390 is the first 390 amino acid residues of diphtheria toxin with an addition of alanine to the N-terminus and two mutations for de-glycosylation. (C): The cartoon structure of A-dmDT390-scfbDb(PSMA) immunotoxin.

For targeted immunotoxin therapy, it is important to determine the response of tumor cells to therapy. It would be useful if the target molecules expressed on the tumor cells could be identified before treatment, and the therapeutic dynamics and mechanisms could be imaged noninvasively during the targeted immunotoxin therapy. In this report, we laid the groundwork for evaluating the targeting specificity and therapeutic potential of the immunotoxin construct A-dmDT390-scfbDb(PSMA) with noninvasive optical imaging.

In this study, A-dmDT390-scfbDb(PSMA) was conjugated to Alexa Fluor 680 dye and used to investigate its utility for tumor-specific imaging and treatment. For this purpose, prostate cancer cells were grown in vitro as monolayer cultures and in vivo as solid tumor xenografts in athymic nude mice. The results confirmed that A-dmDT390-scfbDb(PSMA) immunotoxin had specific tumor targeting property in addition to a distinct and potent antitumor activity against PSMA-positive prostate cancer but not against PSMA-negative prostate cancer.

**2. Results and Discussion**

PSMA is known to be highly expressed and rapidly internalized in malignant prostate cancer cells. However, it is minimally expressed in benign tumors and normal tissues. The PSMA antibody fragment scFv has been used to bind specifically to prostate cancer cells for prostate cancer therapy\[^{21,22}\] and some PSMA-targeted therapeutics have been investigated in clinical trials.\[^{23,24}\] In this study, we demonstrated that the conjugate of a fold-back single-chain diabody of anti-PSMA monoclonal antibody fused to the translocation domains of diphtheria toxin (A-dmDT390-scfbDb(PSMA)) inhibited the growth of PSMA-positive LNCaP cancer cells both in vitro and in vivo but not the growth of PSMA-negative PC-3 cells. The specific toxicity towards LNCaP cells indicated that scfbDb(PSMA) bound specifically to the PSMA antigen on the cell membrane and effectively delivered DT390 into the PSMA-expressing cells to induce cytotoxicity.

**2.1. PSMA is Overexpressed in LNCaP but not in PC-3 Cells**

The expression of PSMA in LNCaP and PC-3 cells was determined with immunofluorescence staining and Western blotting. The results showed high levels of PSMA expression in LNCaP cells, but not in PC-3 cells (Figure 2).

**2.2. A-dmDT390-scfbDb(PSMA) Inhibits Proliferation of LNCaP Cells**

The inhibitory effects of A-dmDT390-scfbDb(PSMA) on the proliferation and viability of LNCaP cells and PC-3 cells were
Fluor 680-labeled immunotoxin were detected with fluorescence microscopy and flow cytometry. As shown in Figure 2, Fluor 680-labeled immunotoxin (green) sequentially. Right: Whole cell lysates from LNCaP and PC-3 cells were analyzed with Western blotting.

Figure 2. Expression of PSMA in prostate cancer cells. Left: LNCaP and PC-3 cells were fixed on slides, and incubated with anti-PSMA antibody and with FITC-labeled secondary antibody (green) sequentially. Right: Whole cell lysates from LNCaP and PC-3 cells were analyzed with Western blotting.

Cells but not in PC-3 Cells

A-dmDT390-scfbDb(PSMA) was labeled with Alexa Fluor 680. The cellular uptakes and accumulations of the Alexa Fluor 680-labeled immunotoxin were detected with fluorescence microscopy and flow cytometry. As shown in Figure 4, the intensity of Alexa Fluor 680 fluorescence increased under microscopy with increasing concentrations of A-dmDT390-scfbDb(PSMA) (from 0.01 μM to 0.1 μM). Similarly, increased red fluorescence was observed in LNCaP cells treated with the labeled A-dmDT390-scfbDb(PSMA) (0.1 μM) when incubated for longer periods (Figure 5). In contrast, no Alexa Fluor 680 fluorescence signal could be detected in PC-3 cells, even after 4 hours of incubation with Alexa Fluor 680-labeled A-dmDT390-scfbDb(PSMA) and at a relatively high concentration (0.2 μM) (Figure S1). Flow cytometry showed that an increase in the concentration and incubation time resulted in more accumulation of labeled A-dmDT390-scfbDb(PSMA) in LNCaP cells (Figure 6). Incubation with labeled A-dmDT390-scfbDb(PSMA) (0.1 μM) for 6 hours caused significant accumulation of the labeled immunotoxin in the LNCaP cells, but not in the PC-3 cells (Figure S2). The heterogeneous distribution of the signal observed under microscopy is largely due to the different stages of cell proliferation and various expression levels of PSMA of the cells.

2.4. A-dmDT390-scfbDb(PSMA) Induces Cell Arrest and Apoptosis of LNCaP Prostate Cancer Cells

LNCaP cells were incubated with graded concentrations (0 to 5 nM) of A-dmDT390-scfbDb(PSMA) for 24 hours and stained with propidium iodide (PI), which binds with DNA. The fraction of cells with sub-diploid DNA content was measured with flow cytometry. As shown in Figure 7A, treatment with increasing doses of unlabeled immunotoxin resulted in dose-dependent increases in the S phase population with concomitant decreases in the G2 phase population of LNCaP cells. However, this effect of the immunotoxin was not evident in PC-3 cells. Similarly, trypan blue dye staining showed significantly increased staining of nonviable cells in LNCaP cultures, but not in PC-3 cell cultures treated with the immunotoxin. The 48 hours DNA content frequency histogram was similar to 24 hour histogram and shown in Figure S3. Light microscopy also showed higher rates of apoptosis in LNCaP cells after incubation with increasing amounts of A-dmDT390-scfbDb(PSMA) (Figure 7B). Fluorescence microscopy showed an increase of green fluorescence signal on the cell membrane due to apoptosis (FITC-labeled annexin V antibody bound with apoptotic cells) when the concentration of the immunotoxin was increased from 0.1 nM to 1 nM. The red fluorescence signal increased (due to DNA binding to PI in necrotic cells) when cells were treated with increased dose of immunotoxin, i.e., from 5 nM and 10 nM immunotoxin (Figure 7B).

Diphtheria toxin exerts its toxicity towards eukaryotic organisms through inactivation of the polypeptide chain EF-2 (Elongation Factor 2). The inactivation results in inhibition of protein synthesis and induction of apoptosis.[25] As a truncated form of diphtheria toxin, DT390, is widely known for inducing cellular toxicity through targeted delivery via a ligand component. The immunotoxins constructed with DT390 are reported to have high toxicity to activated T cells.[14,26] However, little is known about the process of targeted cell apoptosis induced by DT390 in tumor cells. In this study, the immunotoxin of A-dmDT390-scfbDb(PSMA) efficiently induced apoptosis in...
population in LNCaP cell cultures. These may have some implications for the cytotoxicity of the immunotoxin in vitro.

2.5. A-dmDT390-scfbDb(PSMA) Targets LNCaP but not PC-3 Solid Tumor Xenografts in Live Mice

LNCaP cells and PC-3 cells were implanted on the lower left flank of athymic nude mice to test the targeting efficiency of A-dmDT390-scfbDb(PSMA) in prostate tumors. The tumor was allowed to grow to about 5 mm in diameter. Alexa Fluor 680 dye labeled A-dmDT390-scfbDb(PSMA) (100 μL, 200 μg/mL) or Alexa Fluor 680 dye alone was administered as a single bolus via tail vein injection. The whole animal was then imaged at different time points after injection. In this study, near infrared fluorescent imaging was used to confirm the tumor targeting effectiveness of the immunotoxin in LNCaP tumors. Optical imaging provides a dynamic, noninvasive real-time in vivo imaging technique for monitoring the uptake of fluorescent probes in tumor-bearing animals. This optical approach can be used to monitor gene delivery and drug accumulation in tumors.27–29 As shown in Figure 8, accumulation of the fluorescence signal from the labeled immunotoxin was detectable in LNCaP tumors as early as 30 minutes after injection. The fluorescent signal in LNCaP tumors showed an increase during the first 6 hours after injection, followed by a gradual decrease (Figure 8A). The signal from the free Alexa Fluor 680 dye was rapidly detectable throughout the whole body. However, no obvious accumulation of the Alexa Fluor 680 dye was observed in the tumors (Figure 8B). In PC-3 tumor models, both Alexa Fluor 680 labeled A-dmDT390-scfbDb(PSMA) and Alexa Fluor 680 dye showed increased fluorescent signals throughout the whole body during the first 8 hours after injection. However the fluorescent signal showed no obvious targeting to and accumulation in the tumors (Figure S4).

2.6. A-dmDT390-scfbDb(PSMA) Inhibits LNCaP Tumor Growth

The tumor-specific inhibitory effect of A-dmDT390-scfbDb(PSMA) was studied on the PSMA-positive LNCaP tumors and the PSMA-negative PC-3 tumors grown as solid tumor xenografts in athymic nude mice.
in vitro (Figure 3) and in vivo (Figure 9) suggests that the immunotoxin can selectively bind to, and be internalized into, PSMA-expressing cells. This specific targeting in vivo may be responsible for the inhibitory effect of A-dmDT390-scfbDb(PSMA) on the PSMA-positive LNCaP tumor growth, but not on the PSMA-negative PC-3 tumor growth. The findings suggest that this immunotoxin construct has considerable promise and potential for prostate cancer treatment.

Immunotoxins generated by full length anti-PSMA monoclonal antibody [30, 31] or its scFv [32] exhibit specific cytotoxicity and capability to inhibit PSMA-positive prostate tumor growth. However, there are few clinical trials evaluating the clinical potential of immunotoxins linked to PSMA antibody. An important finding of this study was the efficacy of the A-dmDT390-scfbDb(PSMA) immunotoxin against PSMA-positive prostate cancer cells and the lack of binding and cytotoxic effect against PSMA-negative prostate cancer cells. This suggests that inappropriate use of this immunotoxin for treating PSMA-negative tumors may run the risk of unintended systemic toxicity. It is imperative to confirm the presence of PSMA on the tumor cells before initiating therapy with the immunotoxin used in our studies because some prostate cancers do not express PSMA.

3. Conclusion

The A-dmDT390(390)-scfbDb(PSMA) immunotoxin used in this study selectively accumulates in LNCaP cells and tumors that overexpress PSMA. As a consequence of this uptake, LNCaP cells underwent S phase arrest and apoptosis. Intraperitoneal injection of the immunotoxin caused regression of LNCaP solid tumor xenografts in nude mice. Such accumulation and therapeutic response was not seen in PC3 cells and xenografts. This is due to the fact that PC3 cells and tumors are deficient in PSMA expression. Optical imaging experiments confirmed efficient targeted uptake of NIR labeled immunotoxin by PSMA expressing LNCaP cells and solid tumor xenografts in nude mice.

Our study showed that the use of this immunotoxin might be beneficial to individuals with a PSMA-positive prostate tumor. Optical imaging on the individual patient may be processed to determine the expression of PSMA and monitor the efficacy of immunotoxin therapy using the methods described in this paper. This approach will enable us to identify patients who are likely to benefit from treatment with A-dmDT390-scfbDb(PSMA). The fluorescently-labeled immunotoxin is potentially useful for targeted theranostics and personalized medicine.
Figure 7. Cell cycle analysis and apoptosis assay of LNCaP cells after A-dmDT390-scfbDb(PSMA) treatment. (A) LNCaP cells were treated for 24 hours with 0.1, 1, 5, and 10 nM of A-dmDT390-scfbDb(PSMA), respectively, and then stained with PI. Cell cycle distribution was analyzed with flow cytometry. (B) LNCaP cells treated with 0 to 10 nM of A-dmDT390-scfbDb(PSMA) for 48 hours were stained with Annexin V-FITC antibody, washed with buffer and observed under a fluorescent microscope (magnification 400×).

Figure 8. Animal whole body optical imaging after intravenous injection of Alexa Fluor 680-labeled A-dmDT390-scfbDb(PSMA) into LNCaP tumor-bearing mice, showing preferential accumulation of fluorescent signal in tumors. (A) Images obtained after injection of Alexa Fluor 680-labeled A-dmDT390-scfbDb(PSMA) (100 μL) into mice. (B) Images obtained at 0, 1, 3, 6, 12, and 24 hours, respectively, after injection of Alexa Fluor 680 dye alone (100 μL) into mice. The chart on the right shows the changes of the tumor (marked with arrow) to muscle ratio for the fluorescence intensity over time.
Cell Lines: Human prostate cancer cell lines LNCaP and PC-3 were purchased from ATCC (American Type Culture Collection, Manassas, VA). LNCaP and PC-3 cells were maintained as exponentially growing cultures in RPMI 1640 medium and DMEM, respectively. Both media were supplemented with L-glutamine (2 mM), penicillin and streptomycin (50 μg/mL each), and 10% heat-inactivated fetal bovine serum. The cells were maintained in culture at 37 °C and 95% humidity.

Expression and Purification of A-dmDT390-scfbDb(PSMA) Protein: A-dmDT390-scfbDb(PSMA) was expressed in a DT-resistant Pichia pastoris strain. The immunotoxin was produced in shake flask cultures and purified by three purification steps, Phenyl Sepharose hydrophobic interaction chromatography, Q Sepharose anion exchange chromatography and Superdex 200 gel filtration chromatography.[20]

Cellular PSMA Staining: LNCaP and PC-3 cells were plated in 8-well chamber slides with 1 × 10^4 cells in medium (0.5 mL) in each well. After 24 hours, cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and fixed in 3.7% paraformaldehyde. The cells were permeabлизed with 0.2% Triton-X 100 and blocked with 1% bovine serum albumin (BSA) in Hank's buffered salt solution (HBSS) for 1 hour. The cells were then incubated with a rabbit monoclonal anti-PSMA antibody (Abcam, Cambridge, MA) at a dilution of 1:200 to a final concentration of 1 μg/mL. After washing the cells with HBSS, goat anti-rabbit immunoglobulin G conjugated with Alexa Fluor 660 (Invitrogen, Carlsbad, CA) was added to the blocking solution at a final concentration of 2 μg/mL and incubated at room temperature for 1 hour in the dark. The slides were washed three times with DPBS, and examined with a fluorescence microscope.

Western Blot Analysis: LNCaP and PC3 cells were washed with DPBS and lysed with RIPA lysis and extraction buffer (Pierce Biotech, Rockford, IL). Protein concentrations were determined with the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA). Fifty micrograms of proteins were separated by 8% SDS-PAGE electrophoresis and transferred to PVDF membranes. The membranes were blocked using 5% nonfat milk and probed separately for 2 hours at room temperature with primary antibodies for PSMA (Abcam) and β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then washed and probed with a 1:2000 dilution of peroxidase-conjugated secondary antibody, and detected with enhanced chemiluminescence (Amersham Life Sciences, Amersham, UK).

Assessment of Apoptosis: Annexin V binding was used to estimate apoptosis. Cells were first exposed to a DNA-binding dye (PI) to detect sub-diploid population. The two prostate cancer cell lines (LNCaP and PC-3) were treated with 0.1, 0.5, and 1.0 μM of A-dmDT390-scfbDb(PSMA) immunotoxin for 24 hours. The cells were then harvested for trypsinization, collected by centrifugation, and washed with DPBS. The collected cells were fixed with 3.8% formaldehyde before visualization. The fixed cells were stained with PI and Annexin V-FITC and PI. The cells that bound Annexin V-FITC, which had undergone apoptosis, showed green staining on the plasma membrane, whereas

Dye Reagent Concentrate (Bio-Rad, Hercules, CA). Fifty micrograms of proteins were separated by 8% SDS-PAGE electrophoresis and transferred to PVDF membranes. The membranes were blocked using 5% nonfat milk and probed separately for 2 hours at room temperature with primary antibodies for PSMA (Abcam) and β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then washed and probed with a 1:2000 dilution of peroxidase-conjugated secondary antibody, and detected with enhanced chemiluminescence (Amersham Life Sciences, Amersham, UK).

Cell Viability Assays: Cells were seeded in 96-well plates and cultured for 24 hours. The cells were then exposed to a graded range of concentrations of A-dmDT390-scfbDb(PSMA) (from 0 nM to 5 nM) for 48 hours. The viability of cells was measured with a MTT assay. MTT solution (100 μL, 0.5 mg/mL in PBS) was added to each well. The plates were incubated for 4 hours at 37 °C, and then dimethyl sulfoxide (100 μL) was added to each well and incubated for 10 minutes at room temperature. The absorbance was measured at 570 nm using a plate reader. The half maximal inhibitory concentration (IC50) of the immunotoxin was calculated using the SPSS software for performing statistical analysis.

Endocytosis and Quantification of A-dmDT390-scfbDb(PSMA) in Cells: A-dmDT390-scfbDb(PSMA) was labeled with Alexa Fluor 680 using Invitrogen Protein Labeling Kit A20172. LNCaP and PC-3 prostate cancer cells grown to 60 to 70% confluence on four-chamber glass slides were used for endocytosis analysis. The cells were incubated with Alexa Fluor 680-labeled A-dmDT390-scfbDb(PSMA) (0.1 μM) in complete medium for different durations (from 10 minutes to 3 hours), or incubated with graded concentrations of Alexa Fluor 680-labeled A-dmDT390-scfbDb(PSMA) (from 0.01 μM to 0.1 μM) for 3 hours. After removal of the media, the cells were washed three times using DPBS, fixed with 10% formalin for 10 minutes, stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes, and rinsed three times with DPBS. The cells were then observed under fluorescent microscopy. For quantitative measurement of the Alexa Fluor 680-labeled A-dmDT390-scfbDb(PSMA) internalization, a BD flow cytometer (Becton Dickinson, San Jose, CA) with excitation/emission wavelengths of 635 nm/679 nm was used.

Figure 9. Effect of A-dmDT390-scfbDb(PSMA) immunotoxin on LNCaP and PC-3 prostate carcinomas. A-dmDT390-scfbDb(PSMA) (200 μg/kg, 5 μg/mice, per dose) immunotoxin was given via intraperitoneal injection, two doses a day with 6 hour interval for 6 days, compared to equivalent dose of BSA diluted in DPBS for control mice (n = 10 mice per group, *P < 0.1, **P < 0.01). (A) LNCaP tumor volume measurement with MRI. (B) PC-3 tumor volume measurement with MRI. (C) Tumor weight after treatment for 30 days.
the cells that had lost membrane integrity due to necrosis showed red PI staining throughout the nuclei. To further determine the cytotoxicity of A-dmDT390-scfbDb(PSMA), the cells were routinely examined using trypan blue staining and under light microscopy. The cells permeable to trypan blue were considered nonviable.

Animal Tumor Model and Optical Imaging: All animal studies were carried out in accordance with the guidelines of the Howard University Institutional Animal Care and Use Committee. Five-week-old athymic male nude mice were inoculated subcutaneously with $5 \times 10^6$ LNCaP cells or $2 \times 10^6$ PC-3 cells mixed with matrigel (BD Biosciences, Bedford, MA) (0.2 mL) into the lower left flank. The tumors were allowed to reach 5-7 mm in diameter for imaging study. Six mice with LNCaP tumors and five mice with PC-3 tumors were used to test the detection sensitivity and specificity of imaging and the dynamic uptake of Alexa Fluor 680–labeled A-dmDT390-scfbDb(PSMA) in vivo. Three mice with LNCaP tumors and three mice with PC-3 tumors were used to test specific targeting of tumor by Alexa Fluor 680 labeled A-dmDT390-scfbDb(PSMA). Three mice with LNCaP tumors and two mice with PC-3 tumors were given an identical volume of DPBS and served as controls.

In vivo fluorescence imaging of tumor-bearing mice was performed using the IVIS 200 Imaging System and Living Image software (Caliper Life Sciences, Hopkinton, MA). The mice were placed on a warmed (25 °C) stage inside a light-tight camera box with continuous exposure to 2% isoflurane. The mice were given the Alexa Fluor 680 labeled A-dmDT390-scfbDb(PSMA) (100 μL 2.06 μg or 100 μL 4.12 μg) or Alexa Fluor 680 dye via tail vein injection. The mouse was imaged every 10 minutes for the first hour and then imaged every hour for 24 hours. The acquisition time for each image was 2 seconds. Regions of interest around tumor sites from the displayed images were identified and emitted light was measured. The signal intensity was expressed as radiant efficiency in phosphons/second/cm²/steradian/(μW/cm²) ($p/s/cm²/ster/(μW/cm²)$). The contralateral non-tumor-bearing leg muscle was selected as the background.

Evaluation of Therapeutic Efficacy: Tumor xenograft models were developed by subcutaneous inoculation of $5 \times 10^6$ LNCaP cells or $2 \times 10^6$ PC-3 cells mixed with matrigel (0.2 mL) into the lower left flank of five-week-old male athymic nude mice. LNCaP tumors were allowed to develop for six weeks and PC-3 tumors for three weeks to reach the diameter of about 5 mm. The mice with LNCaP tumors or PC-3 tumors were divided randomly into two groups ($n = 10$). A-dmDT390-scfbDb(PSMA) immunotoxin (200 μg/kg) or BSA diluted in DPBS was administered via intraperitoneal injection to twice a day at 6 hour intervals (10 am and 4 pm) for 6 days. Tumor growth was monitored twice a week with MRI. The mice were anesthetized with 2% isoflurane in oxygen, positioned in the MRI probe, and taped with polyurethane foam to avoid involuntary motion. A Bruker 400MHz NMR machine (Bruker-Biospin, Billerica, MA) was used for MRI. A rapid acquisition with refocused echoes (RARE) sequence was used to acquire T2-weighted coronal images. The imaging parameters were: echo time 7.8 ms, RARE factor 16, effective echo time 39 ms, repetition time 3600 ms, number of averages 12, field-of-view 27.0 mm $\times$ 25.6 mm, matrix size $192 \times 256$, and slice thickness 0.5 mm. The Medical Image Processing, Analysis, and Visualization application (CIT/NIH, Bethesda, MD) was used for image analysis. The tumor was manually segmented in each MRI image, the number of voxels within the boundary of tumor was counted and the total tumor volume was calculated.

Statistical Analysis: Statistical analysis was performed with the Student’s t-test. Survival was assessed with the Kaplan–Meier method. A significant correlation was inferred if a P value was $<0.05$ by correlation analysis. All statistical tests were two-sided.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
This work was supported in part by NIH/NCRR 3 G12 RR003048, NIH/NIMHD 8 G12 MD007597, and USAMRMC W81XWH-10-1-0767 grants.

Received: June 30, 2012
Revised: August 22, 2012
Published online:
