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

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Abstract

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-scfbDb(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-scfbDb(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-scfbDb(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-scfbDb(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 (V_H) and the variable light chain (V_L) 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-monkey CD3 bivalent immunotoxin with a fold-back single-chain diabody, A-dmDT390-scFvDb(207), showed a 5- to 7-fold enhanced bioactivity over that containing two tandem unites 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 (scFvDb, Figure 1A) based on the Fv sequences of the murine anti-PSMA antibody J591 and fused it to the C-terminus of DT390 using the approach similar to that for constructing anti-CD3 immunotoxin A-dmDT390-scFvDb(207).^[19] As shown in Figure 1B and C, the bivalent anti-PSMA immunotoxin, A-dmDT390-scFvDb(PSMA), has a sequence of DT390-L1-V_L-L1-V_H-L2-V_L-L1-V_H where L1 is a five-residue linker and L2 is the longer (G₄S)₃ linker, permitting interactions between the distal and proximal V_L/V_H domains. Preliminary studies showed that the scFvDb bound to the extracellular domain of PSMA with a higher affinity than biscFv and single scFv formats at a ratio of 7:2.5:1 (scFvDb:biscFv:scFv). This finding indicates that the scFvDb (PSMA) may be more sensitive and specific for targeted imaging and therapy. Previous *in vitro* studies have further demonstrated that the anti-PSMA fold-back diabody efficiently mediates the entry of the truncated toxin across the cell membrane into the cytosol and the fold-back format immunotoxin is 18- to 30-fold more potent than the biscFv format against monolayer LNCaP cancer cells.^[20]

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-scFvDb(PSMA) with noninvasive optical imaging.

In this study, A-dmDT390-scFvDb(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-scFvDb(PSMA) immunotoxin had specific tumor targeting property in addition to a distinct and potent anticancer 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-scFvDb(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 scFvDb(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-scFvDb(PSMA) Inhibits Proliferation of LNCaP Cells

The inhibitory effects of A-dmDT390-scFvDb(PSMA) on the proliferation and viability of LNCaP cells and PC-3 cells were investigated with a methylthiazolyl tetrazolium (MTT) assay. Cells were treated with various concentrations of A-dmDT390-scFvDb(PSMA) immunotoxin for 48 hours. LNCaP cells were found to be highly sensitive to A-dmDT390-scFvDb(PSMA) immunotoxin, showing an IC₅₀ of 0.57 nM (Figure 3). MTT assay showed 75% killing of the LNCaP cells after 48 hours of treatment. The viability of PC-3 cells was not affected by the immunotoxin, even at a relatively high concentration of 100 nM.

2.3. Cellular Uptake and Accumulation are High in LNCaP Cells but not in PC3 Cells

A-dmDT390-scFvDb(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-scFvDb(PSMA) (from 0.01 μ M to 0.1 μ M). Similarly, increased red fluorescence was observed in LNCaP cells treated with the labeled A-dmDT390-scFvDb(PSMA) (0.1 μ M) 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-scFvDb(PSMA) and at a relatively high concentration (0.2 μ M) (Figure S1). Flow cytometry showed that an increase in the concentration and incubation time resulted in more accumulation of labeled A-dmDT390-scFvDb(PSMA) in LNCaP cells (Figure 6). Incubation with labeled A-dmDT390-scFvDb(PSMA) (0.1 μ M) 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-scFvDb(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-scFvDb(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 PSMA-positive LNCaP cells. Flow cytometry analysis of the cell cycle parameters showed that the immunotoxin caused an increase in S phase population and a decrease in G2 phase 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 μ L, 200 μ g/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-scFvDb(PSMA) Inhibits LNCaP Tumor Growth

The tumor-specific inhibitory effect of A-dmDT390-scFvDb(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. Each mouse was given A-dmDT390-scFvDb(PSMA) (10 μ g) *via* intraperitoneal injection for six consecutive days. A-dmDT390-scFvDb(PSMA) significantly inhibited LNCaP tumor growth while not affecting PC-3 tumor growth (Figure 9A and B, Figure 10). At the end of treatment, the tumors were surgically removed and weighed. The average LNCaP tumor weight in the treatment group was 0.27 ± 0.09 g, which was significantly lower than the tumor weight in the untreated control group (0.67 ± 0.11 g) ($P < 0.05$, Figure 9C). The average weight of the treated PC-3 tumors was not significantly different from the average weight of untreated PC-3 tumors in the control group ($P > 0.05$, Figure 9C). There was no difference for the body weights between the LNCaP tumor-bearing mice and PC-3 tumor-bearing mice, whether or not the animals were treated with the immunotoxin ($P > 0.05$).

Figure 8 shows rapid accumulation of A-dmDT390-scFvDb(PSMA) immunotoxin in LNCaP tumors, whereas no fluorescent signal was detected in PC-3 tumors. High intensity of fluorescent signals from Alexa Fluor 680 labeled A-dmDT390-scFvDb(PSMA) immunotoxin was also observed in the kidneys, suggesting that the immunotoxin or degraded fragments might be excreted through the kidneys. The selective cytotoxicity of A-dmDT390-scFvDb(PSMA) immunotoxin both *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-scFvDb(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-scFvDb(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-scFvDb(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.

4. Experimental Section

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 monolayer 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 with 5% CO₂ in air 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 culture 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 permeabilized 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 (Abeam, 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 680 (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).

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 μ M) 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 μ M to 0.1 μ M) 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.

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 μ M of A-dmDT390-scfbDb(PSMA) immunotoxin for 24 hours. Then, the cells were harvested with trypsinization, collected by centrifugation, and washed with DPBS. The collected cells were fixed with 70% ethanol for 1 hour at 4°C. The fixed cells were washed twice in DPBS, incubated with a solution containing RNase (100 μ g/mL) at 37°C in a water bath for 30 minutes, and then gently re-suspended in PI solution (1 mL, 50 μ g/mL with 3.8 μ M sodium citrate in DPBS). Lastly, the cells were incubated for 15 minutes in the dark at room temperature and analyzed using flow cytometry. The red fluorescence signal from PI staining of individual cells was collected. Ten thousand cells of each sample were counted. For fluorescence microscopy detection, 10,000 LNCaP cells were plated onto an 8-well chamber slide for 24 hours. After incubation with different concentrations of A-dmDT390-scfbDb(PSMA) for 48 hours, the cells were incubated with Annexin binding buffer (500 μ L). Annexin V-FITC (5 μ L) and PI (Abeam) (5 μ L) were added onto the cells, and cells were fixed in 2% formaldehyde before visualization. The fixed cells were examined under a fluorescence microscope using separate filters for FITC and PI. The cells that bound Annexin V-FITC, which had undergone apoptosis, showed green staining on the plasma membrane, whereas 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 5×10^6 LNCaP cells or 2×10^6 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 μ L 2.06 μ M or 100 μ L 4.12 μ M) 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 photons/second/cm²/steradian/(μ Watt/cm²) (p/s/ cm²/sr/(μ W/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 5×10^6 LNCaP cells or 2×10^6 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 μ g/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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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.

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.

Figure 3.

Cytotoxic effect of A-dmDT390-scfbDb(PSMA) on LNCaP cells and PC-3 cells. The viabilities of PSMA-positive (LNCaP) and PSMA-negative (PC-3) cells were determined with MTT assay after 48-hour treatment with A-dmDT390-scfbDb(PSMA). Data represent the mean $\pm$ SD of triplicate determinations.

Figure 4.

Fluorescence microscopy of A-dmDT390-scfbDb(PSMA) internalization in LNCaP cells. Cells were treated with 0.01, 0.05, or 0.1 μM of Alexa Fluor 680-labeled A-dmDT390-scfbDb(PSMA) for 3 hours. After washing with DPBS, cells were fixed and incubated with DAPI, and observed under a fluorescence microscope (magnification 400x).

Figure 5.

Fluorescence microscopy of the time course of A-dmDT390-scfbDb(PSMA) internalization in LNCaP cells. Cells were treated with $0.1 \mu\text{M}$ of Alexa Fluor 680-labeled A-dmDT390-scfbDb(PSMA) for different durations, *i.e.*, 10 min, 30 min, 1 h, and 3 h. After washing with DPBS, cells were fixed and incubated with DAPI, and observed under a fluorescence microscope (magnification 400x).

Figure 6.

Flow cytometry quantification of A-dmDT390-scfbDb(PSMA) accumulation in LNCaP cells. Cells were treated with 0.01, 0.05, or 0.1 μM of Alexa Fluor 680-labeled A-dmDT390-scfbDb(PSMA) for 3 hours (A) or with 0.1 μM of Alexa Fluor 680-labeled A-dmDT390-scfbDb(PSMA) for different durations, *i.e.*, 5, 10, 30, 60, 120, and 180 min (B). After washing with DPBS, cells were collected and analyzed with flow cytometry.

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 400x).

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 μ L) into mice. (B) Images obtained at 0, 1, 3, 6, 12, and 24 hours, respectively, after injection of Alexa Fluor 680 dye alone (100 μ L) into mice. The chart on the right shows the changes of the tumor (marked with arrow) to muscle ratio for the fluorescence intensity overtime.

Figure 9. Effect of A-dmDT390-scfbDb(PSMA) immunotoxin on LNCaP and PC-3 prostate carcinomas. A-dmDT390-scfbDb(PSMA) (200 Jig/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.

Figure 10.

MR images showing the effects of A-dmDT390-scfbDb(PSMA) immunotoxin on LNCaP and PC-3 prostate tumors (marked with *). (A) and (B) are representative MR images through the largest cross section of an untreated control and a treated LNCaP tumor in mice 30 days after treatment. (C) and (D) are MR images of an untreated control and a treated PC-3 tumor.