Photoimmunotherapy targeting prostate-specific membrane antigen; Are antibody fragments as effective as antibodies?

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ABSTRACT
Photoimmunotherapy (PIT) is a highly cell-selective cancer therapy based on an armed antibody conjugate with a phthalocyanine-based photosensitizer, IR700. PIT induces rapid and highly specific necrosis in targeted cancer cells after exposure to near-infrared (NIR) light. Cells not expressing the antigen are not affected. To date, PIT has only been demonstrated with full antibody-IR700 conjugates. In this study, small and bivalent antibody fragments, including anti PSMA diabody (Db) and minibody (Mb) were compared with intact IgG for their effectiveness as PIT agents.

Methods: Radioiodinated antibody and antibody fragments with $^{125}$I were used to determine the timing of maximum binding of each anti-PSMA antibody fragment on the cell surface in vivo in mice bearing either PSMA-positive or -negative PC3 tumors. Then therapeutic efficacy of PIT was examined by exposing mice to NIR light at two time points based on the time of maximum cell surface binding at 6 h post-injection for Db-IR700 and 24 hs post-injection for Mb-IR700 and IgG-IR700, and 24 h after the peak uptake times.

Results: PIT with the same molar concentration of PSMA-Db-IR700, PSMA-Mb-IR700, and PSMA-IgG-IR700 conjugate showed similar therapeutic effects in vitro and in vivo on PSMA-positive PC3 tumor xenografts in cytotoxicity and survival curves (p>0.05).

Conclusion: The use of PSMA-Db-IR700 conjugate results in the shortest time interval between injection and NIR exposure without compromising therapeutic effects of PIT.

Key words: photoimmunotherapy; prostate specific membrane antigen; monoclonal antibody; diabody; minibody; pharmacokinetics
INTRODUCTION
Antibody-based cancer therapies, including therapeutic monoclonal antibodies, antibody-drug conjugates (ADC), antibody-toxin conjugates (immunotoxin) and radio-immunoconjugates, have been a mainstay of molecular targeted therapy (1). The antibodies used in these conjugates typically demonstrate great affinity for the target, however, there are some well-known limitations to the use of whole antibodies. Therapeutic antibodies do not initially permeate across the cell membrane and thus, they induce cytotoxicity at the cell surface typically invoking antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (1). Antibody-drug conjugates or immunotoxins can release their therapeutic payload after binding and internalization (2, 3). Radioimmunoconjugates carry therapeutic radioisotopes that lead to radiation-induced cell death without internalization (4). These approaches have been successful in treating cancers, however, hepatic, renal and bone marrow exposure, often results in dose limiting toxicities.

Photoimmunotherapy (PIT) represents yet another way of harnessing antibodies to direct therapy. In PIT, an antibody is conjugated to a phthalocyanine-based photosensitizer, IR700, and when the conjugate binds the target cell and is exposed to near-infrared (NIR) light, it results in rapid and highly specific target cell death (5). Unlike other immunoconjugates, PIT can limit off-target side effects by two safety check points: it requires the conjugate to be docked at the cell surface and it requires the presence of light to activate. As the conjugate is only effective after binding to target molecules on the cellular membrane, there are few off-target side effects and further safety can be achieved by limiting the region of exposure to NIR light. However, full antibodies, due to their relatively large size, do not penetrate evenly into tumor parenchyma limiting the extent of therapy. Thus, genetically engineered small and bivalent antibody-fragments, which are considerably smaller, might also bind more quickly and stably to the antigen yet improve the intratumoral distribution of the conjugate (6). Therefore, in this study, a small and bivalent anti-PSMA diabody and minibody conjugated to IR700 were compared with an IgG-IR700 conjugate in a PSMA positive tumor model. Timing of maximum binding to PSMA on the cell surface was determined for each antibody type and therapeutic efficacy of PIT with the three agents was compared.

MATERIALS AND METHODS
Reagents
HuJ591, a humanized PSMA specific monoclonal antibody (IgG), was developed at Weill Cornell Medical College and kindly provided by Prof. Neil H. Bander. Anti-PSMA-minibody (Mb) and anti-PSMA-cys-diabody (Db) was kindly supplied by ImaginAb Inc. (Inglewood, CA). IRdye 700-DX NHS ester (IR700) was purchased from LI-COR Biosciences (Lincoln, NE). All other chemicals were of reagent grade.

Synthesis of IR700 conjugated IgG, Mb and Db
IgG (1.0 mg, 6.2 nmol), Mb (0.5 mg, 6.3 nmol) or Db (0.5 mg, 10 nmol) was incubated with IR700 (molar ratio 1:5) in 0.1 M Na2HPO4 (pH 8.6) at room temperature for 30 min, followed by purification with a size exclusion Sephadex G-25M column (PD-10; GE Healthcare, Piscataway, NJ). The concentration of IR700 was calculated by measuring the absorption with a UV-Vis system (model 8453 UV-Visible Value System; Agilent Technologies, Santa Clara, CA) to confirm the number of fluorophore molecules conjugated to IgG, Mb or Db. The number of IR700 per IgG, Mb or Db was 2 - 4 (7).

Radioiodination of IgG, Mb and Db
\(^{125}\text{I}-\text{PSMA-IgG}, \text{Mb, and Db}\) were prepared using the Iodo-Gen procedure. Briefly, 100 µg of each protein (PSMA-IgG, Mb, or Db) was added to each Iodogen coated vial and iodinated with 37 MBq of \(^{125}\text{I}-\text{Na}\) which was neutralized to pH 7.2 with the addition of 0.5 M phosphate buffer at room temperature. After 5 minutes of reaction, each \(^{125}\text{I}\) radiolabeled product was purified with a PD-10 size exclusion column. The specific activities of the radiolabeled IgG, Mb and Db were 5.6 mCi/mg for PSMA-IgG, 6.8 mCi/mg for PSMA-Mb, and 9.4 mCi/mg for PSMA-Db. The radiochemical purity of radiolabeled products was validated with size exclusion-HPLC equipped with a TSK SWxl G3000 column (TosoHaas, Philadelphia, PA), a UV monitor and an on-line radiodetector (Bioscan, Washington, DC). The HPLC was eluted with 0.067 M PBS with 100 mM KCl at a flow rate of 1 mL/min.

Cell culture
A PSMA transfected PC3 cell line, PSMA-positive (PC3pip) cells and a control blank-vector transfected PC3 cell line, PSMA-negative (PC3flu) cells were used for PSMA targeting studies. Both cells were established at the Cleveland Clinic Foundation and kindly provided by Prof. Warren Heston. All cell lines were grown in RPMI 1640 medium (Life
Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (Life Technologies) and 1% Pen-Strep (Life Technologies). All cell cultures were maintained in 5% carbon dioxide at 37°C in a humidified incubator.

**Fluorescence microscopy studies**
PSMA-positive or/and negative cells (1 × 10⁴) were plated on a covered glass-bottomed culture well and incubated overnight. Then, IgG-IR700, Mb-IR700 or Db-IR700 was added to the medium at 10 μg/mL and the cells were incubated for 5 h at 37.0°C in a humidified incubator or for 1 h on ice. Cells were washed once with PBS, and fluorescence microscopy was performed using an Olympus BX61 microscope (Olympus America, Inc., Melville, NY) equipped with the following filters: excitation wavelength 590 to 650 nm, emission wavelength 665 to 740 nm. Transmitted light differential interference contrast images (DIC) were also acquired before and after the fluorescence imaging.

**In vitro PIT**
Cells were seeded into 35 mm cell culture dishes and incubated overnight. Medium was replaced with phenol red free culture medium containing 10 μg/mL of IgG-IR700, 5 μg/mL of Mb-IR700 or 3 μg/mL of Db-IR700 that was the same molar concentration at 70 nM. After incubation for 5 h at 37°C, phenol red free culture medium was added. Then, cells were irradiated with a red light-emitting diode (LED), which emits light at 670 to 710 nm wavelength (L690-66-60; Marubeni America Co., Santa Clara, CA), and a power density of 25 mW/cm² as measured with optical power meter (PM 100, Thorlabs, Newton, NJ). All in vitro experiments were repeated at least three times.

**Phototoxicity assay**
Cytotoxic effects of PIT with IgG-IR700, Mb-IR700 or Db-IR700 were determined with flow cytometric LIVE/DEAD® Fixable Green Dead Cell Stain Kit (Life Technologies), which can detect compromised cell membranes. Cells were trypsinized after treatment and washed with PBS. Green fluorescent reactive dye was added in the cell suspension and incubated at room temperature for 30 min, followed by analysis on a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA).

**Animal tumor model**
All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the local Animal Care and Use Committee. Six- to eight-week-old female homozygote athymic nude mice were purchased from Charles River (NCI-Frederick, MD). During the procedure, mice were anesthetized with isoflurane. PSMA-positive or negative cells (2 × 10⁶/mouse, suspended in PBS) were injected subcutaneously in the dorsum of the mice.

**Biodistribution study**

PSMA-positive and negative tumor-bearing mice were divided into 3-4 groups (n = 3-5) with approximately equal distributions of tumor sizes on the day of study, 5 days after inoculation of the cells. ¹²⁵I-IgG, -Mb or -Db (37 kBq/1.0 μg/200 μL in PBS/mouse) was injected via the tail vein, and the biodistribution of ¹²⁵I-IgG, -Mb or -Db was determined at 6 h, and 1, 2, and 3 days after injection, at 1 and 6 h, and, 1 and 3 days after injection, or, at 1, 6 and 24 h after injection, respectively based on their reported biologic clearance rate. Organs of interest were excised and weighed, and, the radioactivity counts were determined with a gamma counter (Wizard 2480, PerkinElmer, Waltham, MA) using the injected dose as a standard. Data were calculated as the percentage injected dose per gram of tissue (%ID/g).

**In vivo PIT**

PSMA-positive tumor-bearing mice were allocated based on the tumor volume 3 days after inoculation of the cells to each of 3 groups of 9-10 animals per group for the following treatments: (1) no treatment control; (2) the same molar (670 pmol) of agent (100 μg of IgG-IR700, 50 μg of Mb-IR700 or 30 μg of Db-IR700) i.v. (27 pmol/kg), no NIR light exposure; (3) the same molar agent i.v., NIR light administered at 50 J/cm² on day 1 and 100 J/cm² on day 2 after injection of IgG-IR700 and Mb-IR700, or, at 50 J/cm² at 6 h and 100 J/cm² on day 1 after injection of Db-IR700. In order to determine tumor volume, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were measured with an external caliper. Tumor volumes based on caliper measurements were calculated by the following formula: tumor volume = length × (width)² × 0.5. Mice were observed daily, and tumor volumes were measured three times a week until the tumor volume had reached more than 500 mm³ at which time mice had to be euthanized with carbon dioxide.
Statistical analysis
Quantitative data were expressed as mean ± s.e.m. from a minimum of three experiments. Dunnett test was used for multiple comparisons of tumor volumes. The cumulative probability of survival was estimated in each group with the use of the Kaplan-Meier survival curve analysis, and the results were compared with use of the log-rank test. *P* value of < 0.05 was considered statistically significant.

RESULTS
Target-specific, dose-dependent NIR light-induced necrotic cell death in response to PIT
Fluorescence microscopy was performed to confirm target-specific localization of each conjugate. IR700 was mainly localized to the cell membrane after 1 h co-incubation on-ice, and, to the lysosomes of PSMA-positive cells at room temperature. When these cells were observed during continuous NIR light exposure, almost immediate swelling, budding and rupture of the lysosome were observed leading to irreversible cell death (Figure 1). PSMA-positive and negative cells were co-incubated to confirm cell-specific killing. IgG-IR700, Mb-IR700 and Db-IR700 did not localize to PSMA-negative cells which were not killed by exposure to NIR light (Figure 1).
To confirm that rapid phototoxic cell death had occurred, we used the LIVE/DEAD assay, which can detect early cell membrane damage. Death of PSMA-positive cells by PIT with IgG-IR700, Mb-IR700 or Db-IR700 increased in a dose dependent manner with the intensity of NIR light. No cell death was observed in the absence of IgG-IR700 or Mb-IR700 or in the absence of light, while considerable cell death was observed with either unlabeled Db or Db-IR700 without NIR irradiation (Figure 2A). Cell death by PIT with those IR700 conjugates or unlabeled agents was not observed in PSMA-negative cells (Figure 2B).

Biodistribution of ¹²⁵I-IgG, -Mb or -Db
Results of *in vivo* biodistribution studies of ¹²⁵I-IgG, ¹²⁵I-Mb and ¹²⁵I-Db were expressed as % ID/g (Supplemental figure 1) and as ratios to blood (Figure 3, left panels), as a subtraction of PSMA-negative tumor values from PSMA-positive tumor values (Figure 3, center panels) and as ratios of PSMA-negative tumor to PSMA-positive tumor (Figure 3, right panels). Highest activity was observed in the spleen, kidney and, PSMA positive
tumors. Compared with IgG, Db distributed to the kidney immediately after injection. High radioactivity in the stomach at 6 h after injection suggests rapid catabolism. Clearance of Mb was also much faster than IgG; however, there was no evidence of rapid catabolism. The peak difference of %ID/g between PSMA-positive and -negative tumors was at 1 day after injection for IgG and Mb, but was 6 h for Db (Figure 3, center panels).

Tumor growth inhibition in response to PIT
PSMA-positive tumors were treated with PIT using two exposures to NIR light at 50 J/cm² on the peak time of tumor uptake as shown by biodistribution and at 100 J/cm² one day later. In the case of IgG-IR700 or Mb-IR700, the first light exposure took place at 24 h and the second at 48 h after injection whereas for Db-IR700 the first light exposure took place at 6 h and the second on 24 h after injection.
Tumor growth was significantly inhibited in the PIT treatment groups with IgG-IR700, Mb-IR700 or Db-IR700, and significantly prolonged survival was observed in PIT treatment groups with IgG-IR700 or Mb-IR700 (Figure 4). No significant therapeutic effect was observed in the group of each agent only, without NIR irradiation (Figure 4), or, in mice with NIR irradiation only, without injection of agent (Supplemental figure 2). No significant difference on survival was detected between different agents (p>0.05).

DISCUSSION
PIT is a highly selective method of killing cancer cells which depends on exposure to NIR light. Typically a full IgG monoclonal antibody is conjugated with the phthalocyanine dye, IR700. Approximately 24 h after the injection, when the immunoconjugate is bound to the appropriate cell surface targets, exposure to NIR activates the photosensitizer and selectively kills the cells to which the antibody is bound (5, 8-10). Although whole IgGs have the advantage of high input function due to prolonged clearance time from the circulation, antibody fragments such as Dbs and Mbs have theoretical advantages based on their better penetration into tumor resulting in homogeneous micro-distribution. Therefore, this study was conducted to compare the efficacy of PIT using a full antibody and antibody fragments. In order to determine the optimal binding kinetics of antibody fragments to the PSMA expressed on the cell membrane, we employed ¹²³I-labeled antibody fragments in mice bearing both PSMA-positive and PSMA-negative PC3 tumors. Since radioiodine detaches from the cell when radiolabeled fragments are internalized, we could detect the
surface-bound fraction of antibody fragments by comparing PSMA-positive tumor to PSMA-negative controls. Although PSMA is internalized rapidly, IR700 bound to cell membrane and PIT effects were similarly confirmed both at 1 h and 5 h after co-incubation with agents regardless of different internalized amounts of IR700, and the biodistribution data shown in Figure 3 indicated that the optimal binding time for PSMA targeted molecules based on Db, Mb, and IgG was at 6 h, 24 h, and 24 h post injection, respectively. Therefore, for Db-IR700 conjugates NIR exposure in PSMA-positive PC3 tumor-bearing mice was performed at 6 and 24 h, whereas Mb-IR700 and IgG-IR700 conjugates required NIR exposure at 24 and 48 h after injection in the same model. Although all conjugates produced comparable cell killing with PIT, the early treatments with the use of Db-IR700 is a comparative advantage for this antibody fragment. Despite different accumulation doses of the three conjugates, growth suppression effects induced by PIT were similar in all three groups. The higher input function due to longer clearance time of the larger fragments was likely balanced by the improved micro-distribution of the smaller fragments. This 2-time irradiation regimen is considered to improve the efficacy of agent delivery into the tumor, because the first treatment kills tumor cells especially near the vessels, that induces a post-PIT super-enhanced permeability and retention (SUPR) effect which enhances delivery of any macromolecules subsequently administered into the tumor bed. Therefore, we assessed biodistribution of all agents and adopted the first time point as the peak tumor uptake of $^{125}$I and the second as 1 day later when macromolecular intratumoral micro-distribution was optimal due to the PIT-induced SUPR effect. The treatment regimen and irradiation timing for Mb and Db might be further optimized in the clinical setting because the clearance is different in human compared to mice.

As shown in Figure 1, target-specific necrotic cell death was similar in all three groups at 1 and 5 h post-incubation. The Db-IR700 conjugate showed greater selective cytotoxicity to PSMA-positive cells than PSMA-negative cells even without exposure of NIR light at 5 h post-incubation as shown in Fig. 2. However, in vivo cytotoxicity of Db-IR700 conjugate by itself was not observed and cell killing was only seen with NIR exposure. We assessed cytotoxicity of Db and Db-IR700 and found that both of them had dose-related cytotoxicity and the LD50 was c.a.1-3 ug/mL (data not shown), at which level Db saturated cell surface PSMA molecules. However, considering the rapid in vivo clearance of Db, the actual concentration of Db-IR700 in tumor cells in vivo is likely to be far lower than this level. Therefore, all cytotoxicity that was shown in vivo required activation by NIR light.
exposure.
Inaccessibility of light to deep organs is a limitation of PIT even with NIR light, however many tumors such as prostate cancer recurrences or regional lymph nodes would be amenable to PIT using laparoscopic probes. Although LED was used in this study, laser light also can be used and is more effective. Laser can be applied to endoscopy; therefore, PIT might be effective for treatment of disseminated tumor cells and/or tiny recurrences after surgery.

In conclusion, we demonstrate that equally effective PIT can be obtained with both full antibodies and antibody fragments. Because the pharmacokinetics of smaller fragments (e.g. Dbs) are faster, PIT can be initiated at a shorter time interval after smaller fragment injection than with larger fragments and intact antibodies, and this feature may have advantages in clinical translation of PIT. Therefore, the use of Db-IR700 conjugate can minimize the time interval between injection and NIR exposure without compromising the therapeutic effects of PIT despite inferior accumulation of the agent.

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Figure 1. Differential interference contrast and fluorescence microscopy of PIT treated PC3 pip (PSMA-positive) cells or a mix of PC3 pip and flu (PSMA-negative) cells. Cells were incubated with anti-PSMA antibodies (IgG-IR700), Mbs (Mb-IR700) or Dbs (Db-IR700) at 10 µg/mL. Just after fluorescence imaging, only PSMA-positive cells demonstrate necrotic cell death (cell budding/rupture). Scale bar = 50 µm.
Figure 2. Target-specific cell death in response to IgG, Mb or Db-IR700–mediated PIT in PSMA-positive (A) or negative (B) cells. Target-specific cell death in response to PIT was dose dependent to intensity of irradiation. Data are means ± s.e.m. (n ≥ 3).
Figure 3. Analysis of *in vivo* biodistribution of radioactivity after injection of $^{125}$I-PSMA-IgG (A), Mb (B) or Db (C) into mice bearing PSMA positive (PSMA+) and negative (PSMA-) tumors. Each value was calculated using % injected dose/g of tissue (see Supplemental figure 1) for each animal and represented as mean ± s.e.m. ($n = 3 - 5$): the ratios of each organ and tumor to blood (left panels), subtraction of PSMA-negative tumor from PSMA-positive tumor (Center panels), and the ratios of PSMA-positive tumor to PSMA-negative tumor (Right panels) * Value cannot be calculated because some denominators are 0.
Figure 4. Tumor volume and survival curves of PSMA-positive tumor bearing mice. Mice were randomized to one of 3 groups at 3 days after tumor cell injection (n = 9 - 10). IgG-IR700 (A), Mb-IR700 (B) or Db-IR700 (C) was injected, and PIT was performed on 1 and 2 day after injection for IgG-IR700 or Mb-IR700, and at 6 h and 1 day after injection of Db-IR700. Mice were observed until the tumor volume reached more than 500 mm$^3$ at which time mice had to be euthanized. * $P < 0.05$ vs. control, Dunnett multiple comparison test. # $P < 0.05$ vs. control, Log-rank test.
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