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P-glycoprotein Targeted Photodynamic Therapy of Chemoresistant Tumors using Recombinant Fab Fragment Conjugates

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Abstract

P-glycoprotein (Pgp) has been considered as a major cause of cancer multidrug resistance; however, clinical solutions to overcome this drug resistance do not exist despite tremendous endeavors. Lack of cancer specificity is a main reason for clinical failure of conventional approaches. Targeted photodynamic therapy (PDT) is highly cancer specific by combining antibody targeting and locoregional light irradiation. We aimed to develop Pgp-targeted PDT using antibody-photosensitizer conjugates made of a recombinant Fab fragment. We prepared the photosensitizer conjugates by expressing recombinant Fab fragment and specifically linking IR700-maleimide at the C-terminal of the Fab heavy chain. *In vitro* studies showed that the Fab conjugates specifically bind to Pgp. Their phototoxicity was comparable with full antibody conjugates when assayed with conventional 2-D cell culture, but they outperformed the full antibody conjugates in a 3-D tumor spheroids model. In a mouse xenograft model of chemoresistant tumors, Fab conjugates showed Pgp specific delivery to chemoresistant tumors. Upon irradiation with a near-infrared light, they produced rapid tumor shrinkage and significantly prolonged survival of tumor-bearing mice. Compared to the full antibody conjugates, Fab conjugates showed shorter time to reach peak tumor levels and achieved a more homogenous tumor distribution. This allows light irradiation to be initiated at a shorter time interval after the conjugates injection, and thus may facilitate clinical translation. We conclude that our targeted PDT approach provides a highly cancer-specific approach to combat chemoresistant tumors, and that the conjugates made of recombinant antibody fragments are superior to full antibody conjugates for targeted PDT.

Graphical Abstract

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The conjugates of recombinant antibody fragments were developed for Pgp-targeted PDT, providing a highly cancer-specific approach to combat chemoresistant tumors.

Keywords

Antibody conjugates; Cancer targeting; Multidrug resistance; P-glycoprotein; Recombinant antibody fragments; Targeted photodynamic therapy

Introduction

Multidrug resistance (MDR) remains a leading cause for cancer chemotherapy failure.¹ P-glycoprotein (Pgp) mediates active efflux of anticancer agents out of cancer cells and becomes a major cause of cancer MDR.^{2, 3} Pgp was first identified as responsible for chemoresistance in ovarian cancer (OvCa) patients.⁴ Since then, Pgp overexpression has been found to be associated with treatment failure in more than half of human cancers, including ovary, liver and head and neck cancers, as well as leukemia and lymphoma.^{5, 6} Strategies to overcome this resistance have been actively sought for more than 30 years, yet clinical solutions do not exist.^{7, 8} Three generations of small-molecule inhibitors of Pgp have been developed to sensitize MDR tumor cells. However, they have yet to reach the oncology clinic due to lack of cancer specificity.^{9–11}

Antibody-targeted photodynamic therapy (PDT) provides a highly cancer specific approach to treat cancers by combining antibody-based cancer targeting and localized light activation of photosensitizer (PS).¹² Epidermal growth factor receptor (EGFR)-targeted PDT using antibody-photosensitizer conjugates (APCs) is currently tested in Phase I/II trials for the treatment of head and neck cancers (NCT02422979) and has shown promising tumor responses in the patients.¹³ Pgp is an excellent target for antibody-targeted PDT as it is primarily expressed in the plasma membrane of MDR cancer cells for pumping out anticancer agents.⁵ APCs of Pgp antibody can bind to MDR cells specifically and cause Pgp-selective photokilling of these cells after in-tumor light irradiation. Therefore, Pgp-targeted PDT can be highly cancer-specific by combining Pgp antibody targeting and locoregional light irradiation, and thereby overcome the limitation of low cancer specificity that is associated with the conventional small-molecule inhibitor approaches.

Currently, the mainstream APCs for targeted PDT are full-length antibodies that are conjugated to PSs via amine-NHS chemistry.¹² Antibody fragments may be a superior drug carrier for targeted PDT to full-length antibody. Due to the larger molecular weight (150 kDa), full-length antibodies have long circulation half-life and relatively poor tissue penetration, which may limit therapeutic efficacy of PDT and cause photosensitivity in patients for long periods of time.¹⁴ Antibody fragments in smaller size can offer several

advantages over full-length antibodies: (1) faster clearance of small fragments shortens the time to achieve peak tumor levels;¹⁵ (2) antibody fragments have greater penetration into the interior of tumors, leading to a more homogenous distribution; and (3) many small antibody fragments lack Fc-mediated cytotoxicity, avoiding direct damage to normal tissues where the target may also be present.¹⁶ Two recent studies compared small fragments and full antibody as drug carriers for targeted PDT, and reported that small fragments took less time to reach good tumor-to-background (T/B) ratios than full antibody.^{14, 17} Van Driel et al. reported that EGFR-targeted small fragment conjugates achieved greater tumor suppression than full antibody conjugates;¹⁴ whereas Watanabe et al. did not observe greater efficacy of small conjugates.¹⁷ This inconsistency may be due to the random modification method used to link the PSs to the fragments in these two studies.^{14, 17} Currently, all the APCs have been prepared through random modification of lysine residues of the antibodies, which may impair antigen binding and lead to conjugate heterogeneity.¹⁸ Small antibody fragments are less able to tolerate non-specific conjugation because lysine residues in the fragments are more likely critical for target binding, and thus their occupation by the PS may cause dramatic decreases in binding affinity and specificity. Site-specific conjugation of small molecules to antibodies allows precise control over the location of conjugation, and thus yields highly homogeneous materials with improved biological properties.¹⁸ Thus, we speculate that site-specific conjugation methods can yield superior small fragment conjugates for targeted PDT.

In this study, we constructed a Pgp-targeting small antibody conjugates made of a recombinant Fab fragment with a site-specific conjugation approach. We then examined Pgp specificity of the APCs of the Fab and compared their phototoxicity with the full-length antibody conjugates in both conventional 2-D cell culture models as well as 3-D tumor spheroids models. Finally, *in vivo* tumor delivery and anticancer efficacy of the APCs were examined using a mouse xenograft model of drug resistant tumors.

Materials and Methods

Anti-Pgp Antibody Production

Anti-Pgp monoclonal antibody 15D3 (Pab) was produced in-house using the hybridoma cell line from ATCC (Rockville, MD, USA) according to a method described previously.¹⁹ Briefly, hybridoma cells were initially cultured in DMEM media (Corning Inc., Corning, NY, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, USA), and then were adapted into serum-free hybridoma medium (Thermo Fisher Scientific, Rockford, IL, USA). The antibody-containing media was collected and the antibody was purified with a HiTrap Protein G HP column (GE Healthcare Life Sciences, Piscataway, NJ, USA). The identity and purity of the antibody were assessed by SDS-PAGE.

Hybridoma sequencing

Variable regions of the Pab genes were sequenced at Synbuild, LLC (Tempe, AZ, USA). Briefly, total RNA was isolated from the 15D3 hybridoma cells, and reverse-transcribed. The variable regions of the target genes were amplified with a set of proprietary primers from the

cDNA using a standard RT-PCR protocol and sequenced using a standard dye-terminator capillary sequencing method.

Expression and purification of Fab

The heavy and light chain variable domains of the anti-Pgp Fab antibody were fused to the constant domains of a mouse IgG1 and mouse Kappa heavy and light chains respectively using a gene synthesis service (Invitrogen GeneArt Gene Synthesis, Carlsbad, CA, USA) and then subcloned into the eukaryotic-expression vector pαH (Figure S1). Fab variable Kappa light chain (mouse) cloning into PaH vector using kpn1 and bamh1, variable heavy chain (mouse) cloning into PaH vector using kpn1 and bamh1. An HHHHHHC sequence was appended to the C-terminus of the heavy chain. The ExpiCHO transient expression system (Thermo Fisher Scientific) was used to express the anti-Pgp Fab. Briefly, according to the manufacturer's protocols, the plasmids were transfected into ExpiCHO cells at a 70:30 Heavy Chain to Light Chain Ratio. When cell viability fell below 50% the media was harvested and clarified by centrifugation and 0.22 μm filtration. The clarified media was concentrated and buffer exchanged using a tangential flow filtration device into a Ni binding buffer (50 mM NaPO₄ pH 7.2, 500 mM NaCl, 40 mM Imidazole). This sample was then purified using Ni affinity chromatography, eluting in binding buffer with 500 mM imidazole. These fractions were pooled and run over a Superdex 75 size exclusion column that was pre-equilibrated with PBS and 1mM EDTA. Fractions pertaining to the Fab, as evidenced by SDS-PAGE, were pooled and concentrated to 1 mg/ml. A 1 L scale ExpiCHO culture yielded ~4 mg of purified Fab. Five μg of the purified Fab were subjected to SDS-PAGE analysis, either under reducing or non-reducing conditions.

Synthesis of Fab-IR700 and Pab-IR700

Fab-IR700 was prepared by specifically linking IR700-maleimide at the C-terminal of the Fab heavy chain. Pgp-targeting Fab fragment was reacted with IR700-maleimide at molar ratio of 1:2 in phosphate buffer (pH 7.0) containing 1 mM EDTA for 2 h. The resulting conjugates were purified using Zeba™ spin desalting column (40K MWCO, Thermo Fisher Scientific). Pab-IR700 was prepared with a method described previously.²⁰ Briefly, Pab was incubated with IR700-NHS at molar ratio of 1:4 in phosphate buffer (pH 8.0) for 1 h. The product of the conjugation was purified using a Zeba™ spin desalting column (40K MWCO). The protein concentration of the antibody conjugates were determined with BCA protein assay kit (Thermo Fisher Scientific), and the IR700 concentration was quantified by measurement of the absorption at 689 nm with the spectroscopy in order to estimate the number of IR700 molecules conjugated to each antibody molecule. The purity of the APCs was examined by SDS-PAGE. SDS-PAGE was performed with a 4% to 20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA, USA). After electrophoresis, fluorescence intensity on the gel was analyzed with an IVIS Lumina III imaging system (Caliper Life Sciences, Alameda, CA, USA). The gels were then stained with Coomassie Blue Staining buffer (Bio-Rad), and digitally scanned.

Cell lines

The 3T3-MDR1 cell line, a mouse fibroblast cell line stably transfected with a cDNA coding for the human Pgp, was obtained from Dr. Michael Gottesman's laboratory at National

Cancer Institute (NCI). This cell line was maintained in DMEM medium (Corning Inc.) supplemented with 10% FBS (Sigma-Aldrich), 400 IU/mL penicillin, 100 µg/mL streptomycin (Corning Inc.), and 60 ng/ml colchicine (Sigma-Aldrich). KB-8-5-11 is a multidrug resistant human KB carcinoma cell line independently selected with colchicine, which was obtained from Dr. Gottesman's lab at NCI, and were maintained in the same condition as 3T3-MDR1 cell line. The parental cell line KB-3-1 (a subline of HeLa) was also from Dr. Gottesman's lab, and were cultured in the same cell culture medium without colchicine. The human cell lines were characterized by Genetica DNA Laboratories (Cincinnati, OH, USA) using short tandem repeat profiling. GFP and/or firefly luciferase-expressing cell lines were constructed by transfection of the cells with reporter-encoding lentivirus (Biosettia, San Diego, CA, USA) according to a standard protocol provided by the vendor.

Flow cytometry

Immunostaining followed by flow cytometry was performed to detect target specificity of antibody conjugates. Cells were cultured overnight, then trypsinized using 0.25% Trypsin, 0.1% EDTA (Corning, NY, USA), and suspended in PBS buffer. To examine the binding affinity and specificity of antibody conjugates, 1×10^6 of live cells were first blocked with 10% goat serum or 20 µg/ml Pab at room temperature for 10 min, and then stained by Pab-IR700 or Fab-IR700 containing 120 nM IR700 at 4°C for 30 min. Fluorescence of the cells was acquired on an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Ten thousand events of cells were analyzed and the data was processed using FlowJo software (FlowJo, Ashland, OR, USA).

In vitro phototoxicity studies

The phototoxicity of Fab-IR700 was first quantified using Alamar Blue assay according to a method described previously.^{21, 22} Briefly, ten thousand per well of cells were seeded in 96-well plates and cultured overnight. Subsequently, the cells were separately incubated with different concentrations of Fab-IR700 or Pab-IR700 for 4h. Then, drug-containing medium was replaced with fresh medium, and cells were irradiated with the 690 nm LED light (Marubeni America Co., Santa Clara, CA, USA) at 5 J/cm². After 24 h, Alamar Blue reagent (Thermo Fisher Scientific) was added and incubated for 2 h. The fluorescence of the samples was then measured on a Cytation 5 Imaging Reader (BioTek, Winooski, VT, USA) set at 540 nm excitation and 590 nm emission wavelengths.

The phototoxicity of Fab-IR700 was then examined with live/dead staining with a method described previously.^{22, 23} Ten thousand cells were seeded in 96-well plates and were cultured overnight. Medium was replaced with Fab-IR700 or Pab-IR700. The cells were further incubated for 4h at 37 °C. After washing with PBS, the cells were irradiated with a 690 nm LED light at the light dose of 5 J/cm². An hour after NIR irradiation, the cells were co-stained with Calcein AM (2 µM) and PI (5 µg/mL) at room temperature for 30 min, rinsed with PBS, and then imaged using a CYTATION 5 imaging reader (BioTek).

For co-culture study, 3T3-MDR1 and 3T3-GFP cells were co-cultured at ratio of 1:1 at Lab-Tek™ II Chambered Coverglass. Cells were treated with Pab-IR700 or Fab-IR700 (120 nM

IR700) at 37 °C for 4 hrs. After washing with PBS, cells were irradiated with the LED light. After 24 h, the cells were stained with PI, and were then imaged with a CYTATION 5 imaging reader (BioTeK).

For Annexin V and PI staining, 3T3-MDR1 or 3T3 cells were treated with Fab-IR700 or Pab-IR700, and then irradiated with the 690 nm LED light as described above. At 6 h post irradiation, the cells were trypsinized, washed twice, and stained with FITC-labeled Annexin V and PI (BD Biosciences) according to the manufacturer's instruction. The fluorescence of the stained cells was detected with a BD FACS Canto II flow cytometer.

Phototoxicity in tumor spheroids

KB-8-5-11 spheroids were grown to study the photokilling of Pab-IR700 in 3-D model with a method described previously.^{22, 23} Briefly, 1×10^4 of KB-8-5-11 cells in 200 μ L medium per well were seeded into Corning 96 well clear round bottom ultra-low attachment microplate (Corning Inc.), and cultured for 5 days. To evaluate the phototoxicity, KB-8-5-11 spheroids were treated with Fab-IR700 or Pab-IR700 (240 nM IR700) for 4 or 24 h. Then the spheroids were rinsed with fresh medium and irradiated with the 690 nm LED light at the light dose of 10 J/cm². After 24 h, the spheroids were incubated in Calcein AM/PI solution at 37 °C for 30min. After washing, the spheroids were imaged using a Cytation 5 Imaging Reader (BioTeK).

Animals

All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animal Resources (2011, US National Research Council), and approved by the Wake Forest Institutional Animal Care and Use Committee. Female Balb/c nude mice (4–6 weeks old) that were purchased from Charles River (Wilmington, MA, USA) were used in the animal studies.

In vivo tumor delivery and biodistribution

To establish subcutaneous MDR tumor model, 2×10^6 KB-8-5-11-GFP/Luc cells were suspended in 0.1 ml PBS/Matrigel (BD Biosciences) (1/1, v/v) and subcutaneous injected into the right lower flank of Balb/c nude mice; and 2×10^6 KB-3-1-GFP/Luc cells were injected into the left lower flank using the same method. Ten days after tumor inoculation, mice were randomly allocated into three groups and were i.v. injected with PBS, Fab-IR700, or Pab-IR700 (both equivalent to 4 nmol IR700 per mouse). Fluorescence images were taken using an IVIS Imaging System for visualization of IR700 from 1 h post-injection to 24 h post-injection. Twenty-four h after injection, heart, liver, spleen, lung, kidney, and tumors were excised for *ex vivo* IVIS imaging followed by further section.

In vivo tumor response

To examine tumor response, 2×10^6 KB-8-5-11-GFP-Luc cells were suspended in 0.1 ml PBS/Matrigel (1/1, v/v) and injected into nude mice as described previously.²⁴ After tumors formed in a week, mice were i.v. administered with PBS, Fab-IR700, or Pab-IR700 (4 nmol IR700 per mouse). The IR700 distribution was examined with IVIS 2 h after injection of Fab-IR700 and 48 h post injection of Pab-IR700. Right after imaging, the tumors in the Fab-

IR700 groups were exposed to the 690 nm LED light at a total dose of 50J/cm². After light irradiation, tumor growth was measured by a caliper twice per week. Tumor volume was calculated using the following formula: $V = (L \times W^2)/2$, where W is the width and L the length of the tumors measured. Mice were euthanized by carbon dioxide inhalation if any tumor volume exceeds 1000 mm³. The body weight of each mouse will be used as a parameter to evaluate *in vivo* toxicity.

Immunohistochemical analysis

Four days after the NIR irradiation treatment, some animals were sacrificed and tumor tissues were excised for immunohistochemical analyses. Briefly, tumors were collected and fixed in freshly prepared 4% paraformaldehyde for 1 day. Tumor samples were paraffin-embedded, sectioned and stained with H&E. The PCNA staining was performed with primary antibody (PC10, Invitrogen) and Ultra Streptavidin HRP Detection Kit ((Multi-Species, DAB) (Biolegend, San Diego, CA, USA) according to the manufacturer's protocol. Images of the stained sections were taken with Cytation 5 Imaging Reader (BioTeK).

Statistical Analysis

Quantitative data were expressed as mean $\pm$ SD. Means were compared using Student's t test for two-sample comparison or one-way ANOVA followed by Tukey's post-hoc analysis for multiple comparisons. *P* values less than 0.05 were considered statistically significant. Survival analysis was conducted with Kaplan-Meier curves, and their comparison was determined by Log-rank (Mantel-Cox) Test.

Results and Discussion

Fab fragment expression and purification

Using the sequences obtained from an anti-Pgp antibody hybridoma, the heavy and light chain variable domains of the antibody were fused to the constant domains of a mouse IgG1 and mouse Kappa heavy and light chains (Figure 1A), and were then subcloned into the eukaryotic-expression vector pc.H (Figure S1). An HHHHHHC sequence was appended to the C-terminus of the heavy chain (Figure 1B). The plasmids were sequenced before transfected into ExpiCHO cells to produce the Fab. After purification, fractions pertaining to the Fab were collected and analyzed with SDS-PAGE. As shown in Figure 1C, in the non-reduced state there is one dominant band at 45 kDa that corresponds to a disulfide linked Fab dimer while in the reduced lane there are two bands around 25 kDa that correspond to the heavy and light chains, indicating that we have successfully prepared the Fab fragment.

Chemistry of APCs

APCs of the Fab fragment were prepared by specifically linking IR700-maleimide (IR700-Mal, LI-COR) to an additional cysteine residue at the C-terminal of heavy chain, and were named Fab-IR700. After purification, the protein content of Fab-IR700 was measured with BCA assay, and the IR700 concentration was spectroscopically quantitated measured at the wavelength of 689 nm. It is estimated that the conjugates contain approximately 0.9 molecule of IR700 per each Fab fragment. The full antibody conjugates (Pab-IR700) were prepared by random conjugation of lysine residues of the antibody with IR700-N-

hydroxysuccinimide ester (IR700-NHS, LI-COR). Quantitation of protein and IR700 concentrations showed that each antibody molecule contains approximately 2.1 IR700 molecules in Pab-IR700. Thus, for both Pab-IR700 and Fab-IR700, each binding domain contains approximately one IR700 molecule. Fab-IR700 and Pab-IR700 were analyzed with SDS-PAGE. As shown in Figure 2A, Fab-IR700 and Fab were located at the molecular weight site around 45 kDa. After reduced with β -Mercaptoethanol (β ME), the Fab fragment was cleaved to smaller fragments with the molecular weights around 25 kDa. Pab-IR700 and Pab were located around the molecular weight site of 150 kDa, and after reduced with β ME, the antibody was cleaved to heavy chain and light chain, showing two bands at the molecular weights of 50 kDa and 25 kDa, respectively. The IR700 fluorescence distribution of Pab-IR700 and Fab-IR700 matches the Coomassie Blue stain of the SDS-PAGE gel. In addition, free IR700 was not observed in the gel front (Figure 2A, upper). The gel data indicate that the chemical conjugation and purification of Pab-IR700 and Fab-IR700 were performed successfully.

Pgp specificity of Fab-IR700

To evaluate the binding specificity of Fab-IR700 towards Pgp, we performed flow cytometry analyses with 3T3 (Pgp-negative) and 3T3-MDR1 (Pgp-positive) cells after staining with the APCs at 4°C. After incubation with Fab-IR700 or Pab-IR700 at the concentration of 120 nM IR700 at 4 °C for 30 min, the fluorescence of 3T3 cells or 3T3-MDR1 cells was acquired with flow cytometry. As shown in Figure 2B, Pab-IR700 and Fab-IR700 showed 4.9- and 10.5- folded higher binding towards 3T3-MDR1 cells than 3T3 cells, respectively. When 3T3-MDR1 cells were blocked with excess amount of Pab (20 μ g/ml) before Fab-IR700 incubation, we observed a decrease in Fab-IR700 binding, indicating that Fab-IR700 and Pab-IR700 share the same epitope(s) of Pgp on the cell membrane.

In vitro phototoxicity of Fab-IR700

We then examined Pgp-specific phototoxicity of Fab-IR700 in 3T3 and 3T3-MDR1 cells. Equivalent IR700 doses of two APCs were given to the cells to compare their phototoxicity. After incubation of 3T3 and 3T3-MDR1 cells with Fab-IR700 and Pab-IR700 (both containing 120 nM IR700), respectively, followed by light irradiation, we detected the acute phototoxicity of the cells using live/dead staining. As shown in Figure 3A, treatments of both Fab-IR700 and Pab-IR700 produced a quick cell death (only PI signal was shown) in 3T3-MDR1 cells at 4 h after light treatment. However, no cell death was observed when the cells were not irradiated by light, indicating that the sole APCs treatment could not produce cell death. Further, Fab-IR700 or Pab-IR700 treatment produced no phototoxic effects in 3T3 cells (only live cell stain with Calcein AM was observed), indicating the phototoxicity of both APCs is specific to Pgp. To further confirm that the phototoxicity of Fab-IR700 is Pgp specific, we co-cultured 3T3-MDR1 and 3T3-GFP cells, and treated them with Fab-IR700 followed by light treatment. Four hours after light treatment, cells were stained with PI only. Images in Figure 3B and S2 showed PI staining only in 3T3-MDR1 cells but not in 3T3-GFP cells, confirming Pgp specificity of Fab-IR700 mediated photokilling. Thus, our targeted PDT approach can combine Pgp targeting and localized light activation of the PS to enhance cancer specificity for treatment of chemoresistant tumors.

We also evaluated dose-dependent phototoxicity of Fab-IR700, Pab-IR700, and free IR700 in 3T3-MDR1 cells. Free IR700 did not produce any phototoxicity up to the concentration of 400 nM (Figures S3B and S3C), because IR700, a highly charged zwitterion, showed minimal cellular uptake in both 3T3 and 3T3-MDR1 cells (Figure S3A). As shown in Figure 3C, the IC_{50} value of Pab-IR700 was 10.5 nM IR700 and that of Fab-IR700 was 20.1 nM. When the concentration is over 30 nM IR700, two APCs showed similar photokilling of over 80% cell death. Because the molecular weight of full-length antibody (150 kDa) is more than 3-folded higher than the recombinant Fab fragment (45 kDa), Fab-IR700 is more potent than Pab-IR700 when protein concentration is used to calculate the IC_{50} values. Nevertheless, full-length antibody generally shows superior target binding than antibody fragments with only monovalent binding domain.²⁵ In our study, Pab-IR700 was prepared using NHS-Amine conjugation chemistry, and thus the PS may be conjugated to the binding domain of the antibody. On the other hand, in Fab-IR700, the PS is linked to the additional cysteine residue at the C-terminal of heavy chain of the recombinant Fab, which may avoid the occupation of the PS in the binding domain of the Fab and help maintain the binding affinity and phototoxicity of the Fab-IR700 conjugates.

To further examine the cell death events caused by Fab-IR700- and Pab-IR700-mediated PDT, we performed Annexin V (AnV) and PI staining 4 h after PDT treatment to differentiate among viable (AnV^-/PI^- ; lower left quadrant), early apoptotic (AnV^+/PI^- ; lower right quadrant), necrotic (AnV^-/PI^+ ; upper left quadrant), and late apoptotic/secondary necrotic (AnV^+/PI^+ ; upper right quadrant) cells. Analysis of the data in Figure 3D indicates an increase in late apoptotic/secondary necrotic cells after Pgp-targeted PDT treated (80.7% for Pab-IR700 and 85.0% for Fab-IR700) when compared to PBS treated group (2.38% with light treated and 1.84% without light treated) in 3T3-MDR1 cells. At the same time, the viable 3T3-MDR1 cells population decreased from ~97% to 10.5% for Pab-IR700 mediated PDT and 8.52% for Fab-IR700 mediated PDT, while the 3T3 cells were not affected with the same treatment. These results further confirmed that Fab-IR700, as well as Pab-IR700, produced Pgp-specific photokilling and induced a quick cell death.

Phototoxicity in tumor spheroids

We further examined phototoxicity of Fab-IR700 in tumor spheroids which recapitulate some key features of the *in vivo* microenvironment, including hypoxia and the presence of extracellular matrix.^{26–28} In this experiment, we used cancer cell line KB-8-5-11 (Pgp positive) and KB-3-1 (Pgp negative) to establish tumor spheroids. KB-8-5-11 or KB-3-1 tumor spheroids were incubated with Fab-IR700 and Pab-IR700 (both containing 120 nM IR700), respectively, for 4 or 24 h, and then spheroids were irradiated with a 690 nm NIR light. Live/dead staining of the spheroids with Calcein-AM and PI was performed after 24 h culture. As shown in Figure 4, in the 4 h incubation group, Fab-IR700 treated KB-8-5-11 tumor spheroids showed strong PI staining after light irradiation; however, Pab-IR700 treated spheroids only showed overall weak PI staining, and strong staining was only observed at the surface of the spheroids. This difference of the photokilling effects between the two APCs may be due to better penetration of Fab-IR700 in the spheroids than Pab-IR700. As previous studies indicated, antibody fragment showed better penetration in tumors than full-length antibody, because the fragments have smaller molecular size and the lack of

Fc fragment in them reduces the non-specific binding towards Fc receptor in tumor tissue.^{25, 29} However, after 24 h incubation, both Fab-IR700 and Pab-IR700 treated spheroids showed dramatic cell death, indicating that Pab-IR700 could also achieve comparable treatment efficacy when a longer incubation time is given for penetration into the spheroids' cores. Both Fab-IR700 and Pab-IR700 didn't kill KB-3-1 spheroids even after 24 h incubation followed by light irradiation (Figure S4), indicating the photokilling is specific to Pgp-expressing tumor cells in the spheroids.

***In vivo* tumor delivery and biodistribution**

To study *in vivo* tumor delivery and biodistribution of Fab-IR700 and Pab-IR700, a mouse subcutaneous tumor model of chemoresistant cancer was established. At Day 0, 2×10^6 Pgp-expressing KB-8-5-11-GFP/Luc cells were subcutaneously injected into the right lower flank of Balb/c nude mice, while the same amount of Pgp-negative KB-3-1-GFP/Luc cells was injected into the left lower flank (Figure 5A). Ten days after tumor inoculation, PBS, Fab-IR700 or Pab-IR700 (4 nmol IR700 per mouse) was i.v. injected to the nude mice and the accumulation of IR700 was imaged with an IVIS Lumina III system at 1, 2, and 24 h post injection. As shown in Figure 5B, Fab-IR700 started to accumulate in the KB-8-5-11 tumors on the right flank at 1 h post injection, while minimal IR700 accumulation was shown in the left-flank KB-3-1 tumors with low Pgp expression, indicating Pgp specific tumor uptake of Fab-IR700. In the Fab-IR700 treated mouse, we can also observe a significant kidney and bladder accumulation of IR700 (Figures 5B and S5), which indicated a quick renal elimination of Fab-IR700. In comparison, in the Pab-IR700 group, we didn't observe tumor accumulation of IR700 until 2 h post Pab-IR700 injection, and the increase in tumor accumulation continued through 24 h post injection. Liver accumulation of Pab-IR700 was observed 24 h post injection (Figure S5), which indicated that the liver is the main site of full antibody elimination from blood circulation. Renal filtration is a primary clearance pathway of most low molecular weight proteins and peptides.³⁰ Fab fragment has lower molecular weight than full antibody, and thus undergo quick renal filtration.³⁰ Further, the lack of Fc fragment in Fab fragment reduces renal reabsorption and leads to a decreased circulation half-life.³¹⁻³⁴ Quantitative data of FI intensity in Fig 5C showed that Fab-IR700 achieved faster tumor uptake than full antibody conjugates in KB-8-5-11 tumors, with the maximum uptake at 2 h post-injection.

One day after injection, we harvested heart, liver, spleen, Lung, kidney, and both tumors to detect the IR700 distribution *ex vivo*. As shown in Figure 5D, we found that both Pab-IR700 and Fab-IR700 showed a Pgp specific tumor uptake to KB-8-5-11 tumors. The IR700 fluorescence intensity in KB-8-5-11 tumors is 2.55 ± 0.44 fold higher than KB-3-1 tumors for Fab-IR700, and is 2.26 ± 0.21 folded higher for Pab-IR700. During the first 2 h the Fab-IR700 group showed much stronger IR700 accumulation in KB-8-5-11 tumors than Pab-IR700 group; however, it was quickly cleared in the kidney as evidenced by the strong IR700 signal in kidney from 1 to 24 h, and a weaker accumulation of Fab-IR700 than Pab-IR700 at 24 h in KB-8-5-11 tumors (36% of Pab-IR700).

We further sectioned the tumor tissues to study the penetration and localization of Fab-IR700 and Pab-IR700 in tumors. As shown in Figures 5E, S6, and S7, the blood vessels

were stained by Alexa Fluor 594-CD31 (Green), cell nuclei were stained by DAPI (blue), and IR700 was shown in red. For Fab-IR700 treated groups, we observed IR700 signal at deep tissue which is far from the blood vessel (over 100 μm) when compared to Pab-IR700 (Figure 5F), indicating Fab-IR700 has a good tissue penetration ability. This result is consistent with the previous studies in which the smaller size of Fab fragment than full antibody has been frequently used to improve tumor penetration.^{29, 35–37} Moreover, the good penetration of antibody fragments has been utilized for clinical practice as evidenced by the commercial antibody fragments products including CEA-Scan®, Myoscint, and Verluma.^{38, 39}

***In vivo* tumor response**

We further evaluated anticancer effects of our Pgp-targeted APCs in a tumor model of chemoresistant cancers. Mice were divided into three groups, and received i.v. injection of PBS, Fab-IR700, or Pab-IR700 (4 nmol IR700 per mouse). The IR700 distribution was examined with IVIS 2 h after injection of Fab-IR700 and 48 h post injection of Pab-IR700. Both Fab-IR700 and Pab-IR700 showed tumor accumulation in the orthotopic tumor sites (Figure 6A). Right after the fluorescence imaging, the tumors were irradiated with the 690 nm NIR light, tumor size was measured by a caliper. The results in Figure 6B showed that targeted PDT significantly suppressed the tumor growth when compared with the PBS group. The median survival time of the PBS group was 20.5 days, while that of the Fab-IR700 group and Pab-IR700 group were 30 days and 31 days, respectively (Figure 6C), indicating that targeted PDT can extend the survival days of MDR tumor-bearing mice. We collected the tumor tissues at Day 11 for histological observation. Images from H&E staining and PCNA staining showed that targeted PDT using Fab-IR700, as well as Pab-IR700, disrupted the complexity of tumor tissues and inhibited tumor cells proliferation (Figure 6D). The body weights of mice are shown in Figure S8, indicating there was not weight loss of the mice after targeted PDT procedures.

Targeted PDT using our Fab fragment conjugates achieved excellent tumor response. In a previous study, prostate-specific membrane antigen-targeted PDT using full antibody conjugates achieved superior tumor response to the small fragment conjugates, which showed weak tumor responses in tumor survival studies.¹⁷ Although multiple causes can lead to greater performance of our Fab fragment conjugates, application of site-specific conjugation approach in this study may have an advantage in maintaining the binding affinity of small antibody fragment over the random conjugation approach used in the previous study.¹⁷ In this study, targeted PDT using Fab-IR700 and Pab-IR700 at the same IR700 doses achieved similar tumor responses as shown in Figure 6. Although Fab conjugates show greater tumor penetration than full antibody conjugates in the previous studies^{29, 35–37} and our study (Figures 5E and 5F), their short circulation half-life may lead to sub-optimal overall tumor uptake. On the other hand, full antibody conjugates have long circulation half-life, which may compensate their relatively slow tumor penetration with greater overall tumor uptake. This phenomenon was observed in our study with tumor spheroids model (Figure 4). Targeted PDT with Pab-IR700 showed greater photokilling towards the spheroids after 24 h incubation with the APC than 4 h incubation, whereas Fab-IR700 achieved similar photokilling after 4 and 24 h incubation (Figure 4). In spite of the

similar anticancer activity of the two APCs, Fab-IR700 showed shorter time to achieve the peak tumor level and a reasonable T/B ratio to perform PDT. As shown in Figure 6A, Fab-IR700 achieved excellent tumor uptake and relatively low uptake in normal tissues at 2 h post injection in this orthotopic tumor mouse model, while Pab-IR700 achieved that at 48 h post injection. The quick tumor uptake and fast systemic clearance of Fab-IR700 allow light irradiation to be initiated at a shorter time interval after the conjugates injection. This is important to perform targeted PDT in clinical setting, because long time interval between injection and irradiation would result in prolonged stay of the patients in the hospital and this also can increase the possibility of phototoxicity to the patients.¹⁷

Pgp is not the single mediator of cancer drug resistance, and heterogeneous Pgp expression in tumors is a barrier for clinical translation of Pgp targeted therapeutic approaches.⁷ However, our approach described here can help overcome this barrier. The anticipated success of our targeted PDT approach will depend on a companion diagnostic test designed to determine precisely whether a patient will benefit from the specific treatment. Although this study focuses on cancer therapy, the results of fluorescent imaging in Figures 5 and 6 indicated that the Fab-IR700 conjugates can also be used to image tumoral Pgp and lead to theranostic approach to tackle Pgp in tumors. To further address heterogeneity of Pgp expression in tumors, we can combine our Pgp targeted PDT with chemotherapy, with targeted PDT to eliminate Pgp-positive MDR cells and chemotherapy agents to treat Pgp-negative tumor cells. We mainly focus on enhancing cancer specificity with our Pgp-targeted approach in this study, and this will build up a good foundation for future work involving combination with other modalities.

Conclusion

In conclusion, our Pgp-targeted PDT provides a highly cancer-specific approach to combat chemoresistant tumors by combining antibody targeting and locoregional light irradiation, and thus can overcome the limitation of low cancer specificity that is associated with the conventional small-molecule inhibitor approaches. We have prepared two APCs for targeted PDT: small antibody conjugates made of a recombinant Fab fragment with a site-specific conjugation approach, and full antibody conjugates prepared with a conventional conjugation chemistry. *In vitro* studies showed that both APCs specifically bind to Pgp, and their Pgp specific phototoxicity is similar when assayed with conventional 2-D cell culture. But Fab-IR700 outperforms the full antibody conjugates in a 3-D tumor spheroids model. When tested using a mouse xenograft model of chemoresistant tumors, both APCs showed Pgp specific delivery to chemoresistant tumors. Upon irradiation using a 690 nm near-infrared light, they produced rapid tumor shrinkage and significantly prolonged survival of chemoresistant tumor-bearing mice. Compared to the full antibody conjugates, the small Fab fragment conjugates showed shorter time to achieve peak tumor levels and a more homogenous tumor distribution. This allows light irradiation to be initiated at a shorter time interval after the conjugates injection, and may facilitate clinical translation of targeted PDT. Therefore, the conjugates made of recombinant antibody fragments using site-specific conjugation approach is superior for targeted PDT to conventional full antibody conjugates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Expression and purification of recombinant Fab fragment. (A) Structures of full-length antibody and recombinant Fab fragment. (B) Constructs of the heavy chain and light chain of the recombinant Fab. An HHHHHHC sequence was appended to the C-terminus of the heavy chain. (C) SDS-PAGE of the recombinant Fab fragment under reduced or non-reduced state. A dominant band at 45 kDa in the non-reduced state corresponds to a disulfide linked Fab dimer, while the bands around 25 kDa in the reduced state correspond to the heavy and light chains of the Fab fragment.

Figure 2.

Purity and binding specificity of the APCs. (A) Pgp-targeted Fab fragment and Fab-IR700 were separated by SDS-PAGE in reduced (left two lanes) and non-reduced conditions (right two lanes). Prior to the Coomassie stain (lower gel), the fluorescence of the IR700 was detected (depicted in red in the upper gel). (B) Flow cytometry analysis of Pgp expressing cell line 3T3-MDR1 (up) or Pgp negative cell line 3T3 (down) after treatment with Pab-IR700 or Fab-IR700. For competition assay, 3T3-MDR1 or 3T3 cells incubated with Pab prior to treat with Fab-IR700.

Figure 3.

Phototoxicity of Fab-IR700 and Pab-IR700. (A) Live/Dead staining of 3T3-MDR1 cells with or without light irradiation or 3T3 cells with light irradiation after treated with Pab-IR700 or Fab-IR700. Scale bar, 1000 μ m. (B) PI staining in mixed 3T3-GFP and 3T3-MDR1 cells after Fab-IR700 mediated PDT. Scale bar, 1000 μ m. (C) Dose-dependent phototoxicity of Pab-IR700 and Fab-IR700 in 3T3-MDR1 cells. Data are means $\pm$ SD (n = 3). (D) Flow cytometry analysis of 3T3 and 3T3-MDR1 cells after targeted PDT followed by Annexin V-FITC and PI staining.

Figure 4.

Phototoxicity of Fab-IR700 and Pab-IR700 in KB-8-5-11 tumor spheroids. The tumor spheroids of KB-8-5-11 were treated by Fab-IR700 and Pab-IR700 for 4 (left) or 24 h (right). After washing, the spheroids were irradiated with the 690 nm LED light. Live/dead staining of the spheroids with Calcein AM and PI was performed after 24-h culture. Scale bar, 300 μm.

Figure 5.

Tumor uptake and biodistribution of Fab-IR700 and Pab-IR700 in a mouse xenograft model of chemoresistant tumors. (A) The scheme of the animal model. Mice were inoculated with two different tumor cells in each lower flank. The left lower flank tumor is KB-3-1 tumor with low Pgp expression, while the right lower flank tumor is KB-8-5-11 tumor with high Pgp expression. (B) IVIS imaging of tumor uptake for Pab-IR700 and Fab-IR700. BLI showed that the tumor loading in two flanks of the mice were similar. FI showed Fab-IR700 achieved faster tumor accumulation than full antibody conjugates in KB-8-5-11 tumors, with the maximum uptake at 2 h post-injection. (C) Quantitative FI data of KB-8-5-11 tumor accumulation. (D) Biodistribution of two conjugates at 24 h post injection. Fab-IR700 showed higher tumor delivery towards Pgp-expressing KB-8-5-11 tumors. Pab-IR700 was accumulated in the liver, while Fab-IR700 was deposited in the kidney. (E) Confocal images of intratumoral distribution of Fab-IR700 and Pab-IR700 in KB-8-5-11 tumors 24 h post

injection. The scale bar is 20 μm . (F) Quantitative analysis of distribution of Pab-IR700 and Fab-IR700 in KB-8-5-11 tumors. Distance was calculated from the CD31 stained blood vessel.

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Figure 6.

In vivo tumor response of targeted PDT. (A) IVIS imaging of tumor uptake of Fab-IR700 (2 h post I.V. injection) and Pab-IR700 (48 h post I.V. injection). (B) Tumor growth suppression by targeted PDT with Fab-IR700 and Pab-IR700 at the same IR700 dose. Suppression of KB-8-5-11 tumor growth was observed after Fab-IR700 and Pab-IR700 mediated targeted PDT. Data are presented as mean $\pm$ SD ($n = 6$, ** $p < 0.005$). (C) Kaplan-Meier survival curve of Pab-IR700 and Fab-IR700 mediated targeted PDT in KB-8-5-11-GFP-Luc MDR tumor model ($n = 6$, ** $p < 0.005$). (D) Histological observation of KB-8-5-11 tumors using H&E staining and PCNA staining. Scale bar, 100 μ m.