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Photoimmunotherapy of Prostate Cancer With Antibody and Fab Fragments Targeting the Prostate Specific Membrane Antigen

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Abstract. *Background/Aim: The standard treatment for localized prostate cancer involves surgical removal of the prostate with curative intent. However, when tumor cells persist in the operation site, there is high risk of local recurrence and tumor spread, leading to stressful follow-up treatments, impaired quality of life, and reduced overall survival. This study examined photoimmunotherapy (PIT) as a new treatment option for prostate cancer cells. Materials and Methods: We generated conjugates consisting of either a humanized antibody or Fab fragments thereof targeting the prostate specific membrane antigen (PSMA), along with our silicon phthalocyanine photosensitizer dye WB692-CB1. PSMA-expressing prostate cancer cells were incubated with the antibody dye or Fab dye conjugates and cell binding was measured using flow cytometry. Cells were irradiated with varying doses of red light for dye activation, and cytotoxicity was determined by erythrosin B staining and subsequent analysis using a Neubauer counting chamber. Results: Specific cytotoxicity was induced with the antibody dye conjugate in the prostate cancer cells in a light dose-dependent manner. Treatment of the cells with the Fab dye conjugate resulted in lower cytotoxicity, which could be attributed to a reduced binding affinity and a reduced dye uptake of the Fab fragment. Conclusion: Our new antibody dye and Fab dye conjugates offer potential for future intraoperative PIT in patients with localized prostate cancer, with the aim to ensure complete removal of tumor cells from the surgical area, to avoid local recurrence, and to improve clinical outcome.*

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More than 1.4 million men are diagnosed with prostate cancer (PC) worldwide every year and more than 375,000 die from it (1). Recent advances in detection and diagnosis help to recognize the tumors at an early stage. Around 90% of diagnosed prostate tumors have not yet spread at the time of diagnosis. In this situation, surgical removal of the prostate (radical prostatectomy, RP) is the standard treatment with curative intent (2). Especially, if tumor infiltration to surrounding tissues was found during RP or if there is a risk of damaging adjacent tissues during surgery, tumor cells may remain in the surgical area and cause recurrence. Indeed, approximately 20-40% of patients develop local or systemic recurrence of PC within five years after RP (3). In advanced stages, patients are treated with chemo-hormonal therapy or radiotherapy (4, 5), which are accompanied by severe side effects and impaired quality of life (QoL) (6). In metastasized PC, the 5-year overall survival (OS) rate drops from approximately 100% for patients, who were cured by RP, to only approximately 30% (7). New therapeutic approaches are therefore urgently needed for the targeted removal of residual tumor cells in the operation field.

Photoimmunotherapy (PIT) may represent such a new therapeutic option. In this approach, cytotoxic fluorescent

dyes, which act as photosensitizers, are coupled to tumor-specific antibodies. The antibody dye conjugates bind specifically to the tumor antigens on cancer cells and are internalized. The dye can be activated by irradiation with visible light and trigger cell death *via* photochemical reactions (8). We have designed and synthesized the silicon phthalocyanine photosensitizer WB692-CB1, which can be coupled *via* a maleimide linker to free cysteines that have been incorporated into tumor-specific antibodies. In the present study we coupled WB692-CB1 to a cysteine-engineered antibody and Fab fragments thereof targeting the prostate specific membrane antigen (PSMA) and evaluated the PIT with the conjugates on PC cells.

Materials and Methods

Cell lines. The PC cell lines LNCaP, C4-2, PC3 (ATCC, Manassas, VA, USA) and PC3-PSMA (kindly provided by P. Giangrande, University of Iowa, Iowa City, IA, USA) were cultivated in RPMI1640 medium (Gibco, Invitrogen, Karlsruhe, Germany), 10% fetal calf serum (Sigma Aldrich, St. Louis, MO, USA) and penicillin/streptomycin (100 U/ml, 100 mg/l) at 37°C and 5% CO_2. Cell line identities were verified using short tandem repeat (STR) analysis (CLS GmbH, Eppelheim, Germany).

Generation of the antibody dye and Fab dye conjugates. The humanized IgG1 anti-PSMA antibody h3/F11$^{A118C/D265C}$ containing the cysteine mutations A118C and D265C (EU numbering) in the heavy chains was kindly provided by Heidelberg Pharma AG (Ladenburg, Germany) and dissolved as 1 mg/ml solution in 1 mM EDTA in PBS (pH 7.4). The phthalocyanine dye WB692-CB1 was designed and synthesized by us and dissolved at a concentration of 1 μg/μl in DMSO. For dye conjugation, the antibody was reduced in 40-fold equimolar Tris-(2-carboxyethyl)-phosphine-hydrochloride (TCEP) for 3 h followed by overnight dialysis against 1 mM EDTA in PBS (pH 7.4) and re-oxidation with 30-fold equimolar dehydroascorbic acid (Sigma-Aldrich) for 4 h on a shaker. For the removal of unbound dye 25-fold equimolar N-acetyl-L-cysteine was added and the sample was incubated on the shaker for additional 15 min. After overnight dialysis against PBS, the antibody dye conjugate h3/F11$^{A118C/D265C}$-WB692-CB1 was purified using a HiTrap™ Protein G column (Cytiva, Freiburg, Germany) according to the manufacturer's instructions and dialyzed against PBS. For the generation of the Fab dye conjugate Fab^{A118C}-WB692-CB1, the antibody dye conjugate h3/F11$^{A118C/D265C}$-WB692-CB1 was digested with papain using the Pierce Fab Preparation Kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations of the conjugates were determined using the Pierce BCA™ Protein-Assay (Thermo Fisher Scientific).

SDS-PAGE and western blot analyses. Antibodies and Fab fragments were analyzed before and after dye conjugation by SDS-PAGE under reducing conditions according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). For detection of the coupled dye, gels with the conjugates were viewed under red light (680 nm) using an IVIS 200 Imaging System (Spectral Instruments, Tuscon, AZ, USA). For western blot analyses, target cells were lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% NaDeoxycholate, 0.05% SDS, 1% Igepal. Protein concentrations were determined using the Quick Bradford Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). 50 μg lysate per lane were blotted onto nitrocellulose membranes. PSMA expression was detected using the anti-PSMA mAb K7 (9) and horse radish peroxidase (HRP)-labeled polyclonal rabbit anti-mouse IgG (Dako, Hamburg, Germany). β-actin was detected as loading control using the HRP-conjugated β-Actin rabbit pAb (Cell Signaling Technology Europe, Leiden, the Netherlands). Western blots were developed with an enhanced chemiluminescence (ECL) system and protein bands were analyzed using an INTAS Chemo Star Imager (INTAS Science Imaging Instruments, Göttingen, Germany).

Flow cytometry. Binding of the antibodies and Fab fragments before and after dye conjugation was measured by flow cytometry as described (10). Goat-anti-human Ig(H+L)-RPE (Southern Biotech, Birmingham, AL, USA) was used as detection antibody. Binding affinities were calculated using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA) and dissociation constants (K_D) were determined as the antibody, Fab or conjugate concentrations leading to half-maximal specific binding.

Photoimmunotherapy. Target cells were seeded at a concentration of 2.5×10^5 cells in 2 ml medium per 35 mm^2 petri-dish overnight and incubated with 10 μg/ml h3/F11$^{A118C/D265C}$-WB692-CB1 or with an equimolar concentration of Fab^{A118C}-WB692-CB1. Control cells remained untreated or were incubated with equimolar amounts of uncoupled antibody, uncoupled Fab fragments or free dye. After 24 h, medium was exchanged and cells were irradiated with different doses of red light (λ=690±10 nm) from a LED lamp (L690-66-60; Marubeni America Co., Dinslaken, Germany) ranging from 6 to 32 J/cm^2. Twenty-four h after irradiation, living cells were trypsinized, stained with erythrosine B (Logos Biosystems, Gyeonggi-do, Republic of Korea) and counted using a Neubauer Counting Chamber. Mean values±SD from three independent experiments were calculated using GraphPad Prism 7 software. *p*-Values were determined using the unpaired *t*-test with Welch's correction.

Results

The anti-PSMA antibody h3/F11$^{A118C/D265C}$ (mAb) with two cysteine modifications at positions A118C and D265C in the C_H1 and C_H2 domains of the heavy chain, respectively, was coupled with our silicon phthalocyanine photosensitizer dye WB692-CB1 (Figure 1A and B). SDS PAGE revealed that the antibody dye conjugate h3/F11$^{A118C/D265C}$-WB692-CB1 (mAb-conjugate, Figure 1C) was obtained in high purity after Protein G affinity chromatography and that the dye was successfully coupled to the heavy chains of the antibody (Figure 1D).

The PC target cells were analyzed for PSMA expression using western blot. As shown in Figure 2A, the cell lines LNCaP, C4-2 and PC3-PSMA were found to be PSMA positive and the control cell line PC3 to be PSMA negative. Specific binding of the mAb and the mAb-conjugate was detected on PSMA-expressing LNCaP cells. For the mAb a mean K_D of 0.63±0.10 and for the mAb-conjugate a comparable mean K_D of 0.56 μg/ml±0.07 were calculated (Figure 2B). No binding of both was found on PSMA-negative PC3 cells (Figure 2C).

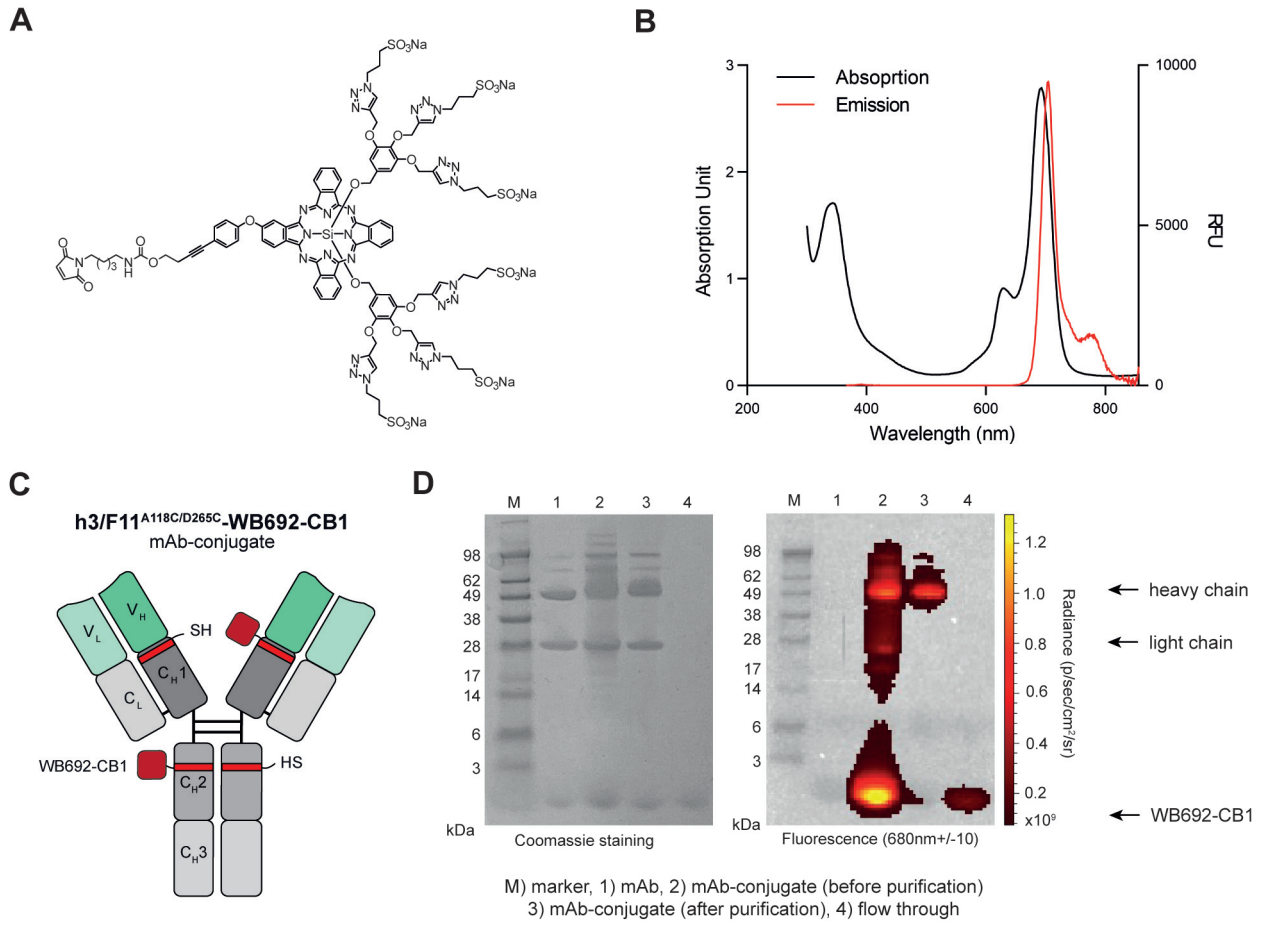

Figure 1. *Generation of the mAb-conjugate h3/F11$^{A118C/D265C}$-WB692-CB1. (A) Chemical structure of the photosensitizer dye WB692-CB1. (B) Absorption and emission spectra of WB692-CB1 (Abs λ_{max}=693 nm/Em λ_{max}=703 nm). (C) Schematic illustration of the mAb-conjugate h3/F11$^{A118C/D265C}$-WB692-CB1 with the mutated cysteines at positions A118C in the C_H1 domain and D265C in the C_H2 domain, respectively, presenting free SH groups for dye coupling. (D) SDS gel of the mAb h3/F11$^{A118C/D265C}$ and the mAb-conjugate h3/F11$^{A118C/D265C}$-WB692-CB1 before and after purification via affinity chromatography. SDS gel stained with Coomassie blue (left) for the detection of the antibody chains and under red light for the detection of the coupled WB692-CB1 dye (λ=680 nm, right).*

The mAb-conjugate elicited a light dose-dependent reduction of PC cell viability. For example, viability of LNCaP cells was significantly reduced to 46.2±15.2% after irradiation with 16 J/cm^2 (p=0.0256) and to 13.7±7.6% after irradiation with 32 J/cm^2 (p=0.0026, Figure 2D). LNCaP cells that were incubated with the conjugate, but were not irradiated with light (0 J/cm^2), remained alive. Moreover, cells that were incubated with the mAb or with free dye were also not affected by irradiation even at a light dose of 32 J/cm^2 (Figure 2E). Similar results were obtained after PIT with the mAb-conjugate in C4-2 and PC3-PSMA cells with highest cytotoxicity in PC3-PSMA cells (p<0.0001, Figure 2F-I). PSMA-negative PC3 cells remained unaffected by PIT with the mAb-conjugate, even at the highest dose of 32 J/cm^2 (Figure 2J and K).

Digestion of the mAb-conjugate with papain led to the high purity Fab^{A118C}-WB692-CB1 conjugate (Fab-conjugate) (Figure 3A and B). Coupling of the WB692-CB1 dye to the Fab fragments reduced their binding affinity about 22-fold from 0.34±0.13 μg/ml to 7.68±2.00 μg/ml (Figure 3C). Specificity of Fab fragments was not impaired by dye conjugation, because no binding of the conjugates to PSMA-negative PC3 cells was detected (Figure 3D). Incubation of LNCaP cells with the Fab-conjugate followed by irradiation with a light dose of 32 J/cm^2 significantly reduced the viability to 59.9±19.6% (p=0.0305, Figure 3E).

Discussion

We could show that our new silicon phthalocyanine dye WB692-CB1 can be coupled *via* its maleimide linker to a cysteine engineered anti-PSMA antibody and be used as photosensitizer for the PIT of PC. In the past, dyes for the

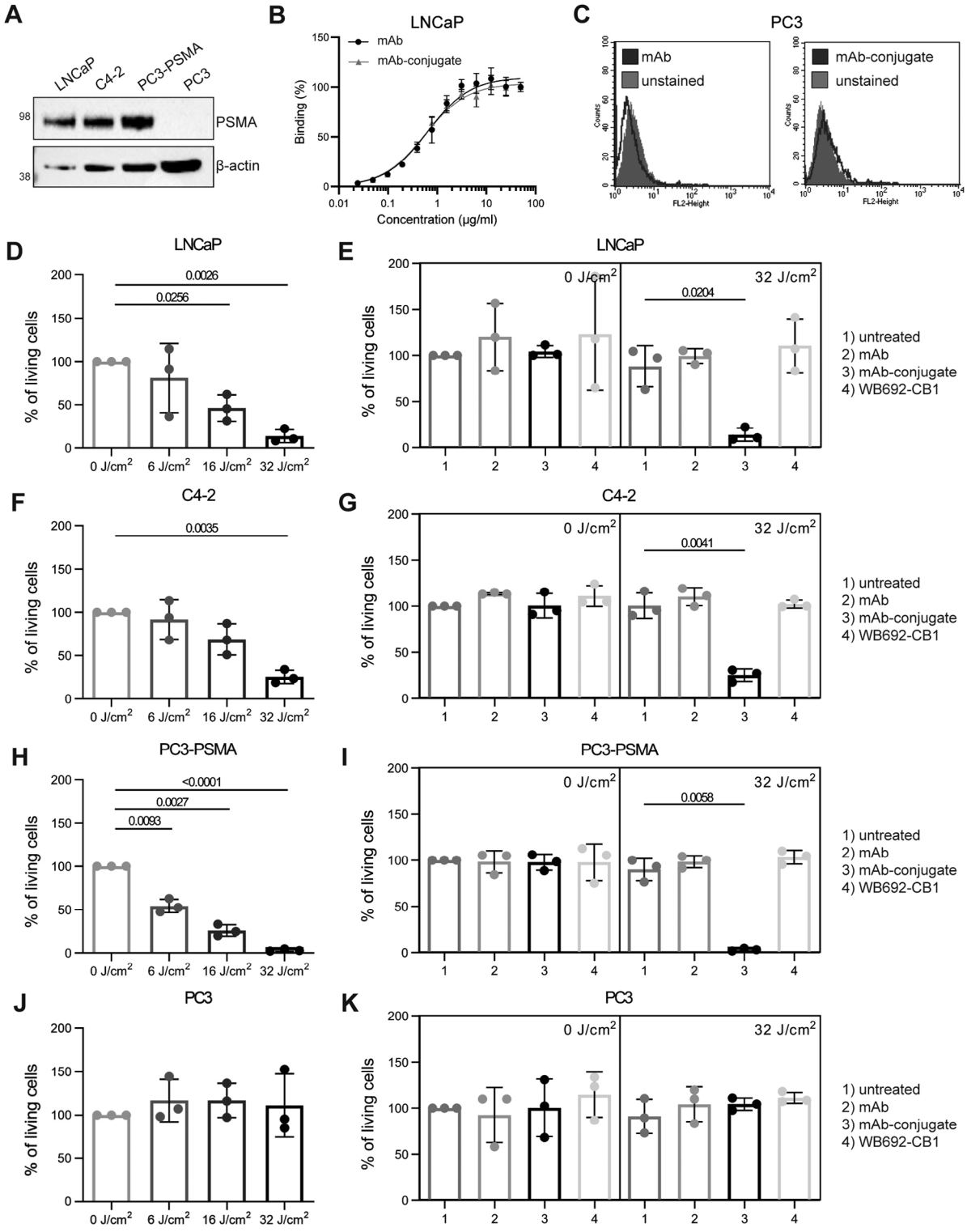

Figure 2. *Photoimmunotherapy of PSMA expressing PC cells with the mAb-conjugate h3/F11$^{A118C/D265C}$-WB692-CB1 using different light irradiation doses. (A) PSMA expression of the PC cell lines as shown by western blot. β-actin was detected as loading control. (B) Binding of the mAb h3/F11$^{A118C/D265C}$ and the mAb-conjugate h3/F11$^{A118C/D265C}$-WB692-CB1 to PSMA-positive LNCaP and (C) PSMA-negative PC3 cells. Viability of the PC cell lines (D, E) LNCaP, (F, G) C4-2, (H, I) PC3-PSMA and (J, K) PC3 after incubation with the mAb-conjugate and irradiation with different red-light doses (λ=690±10 nm). Cell viability was determined using erythrosin B staining and a Neubauer counting chamber. Mean values±SD from three independent experiments. p-Values were determined using the unpaired t-test with Welch's correction.*

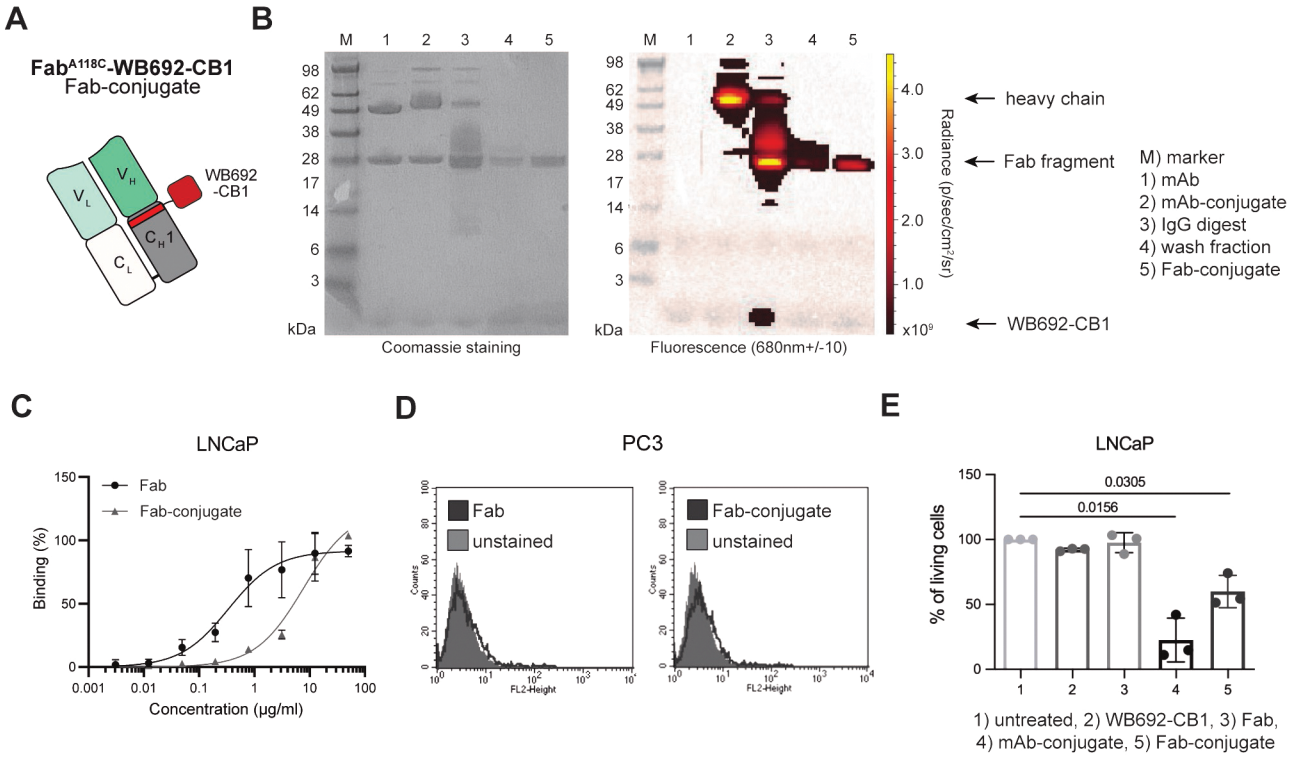

Figure 3. *Photoimmunotherapy of PC cells with the Fab-conjugate Fab$^{A118C/D265C}$-WB692-CB1. (A) Schematic illustration of the Fab-conjugate. (B) SDS gel of the mAb, the mAb-conjugate and the digested Fab-conjugate before and after purification via affinity chromatography. SDS gel stained with Coomassie blue (left) and under red light (λ=680 nm, right). (C) Binding of the uncoupled Fab fragment and the Fab-conjugate to PSMA-positive LNCaP and (D) PSMA- negative PC3 cells. (E) Viability of LNCaP cells after incubation with the Fab-conjugate and irradiation with 32 J/cm^2 red light (λ=690±10 nm). Control cells were incubated with the mAb-conjugate, uncoupled Fab fragment or free dye. Cell viability was determined using erythrosin B staining and a Neubauer counting chamber. Mean values±SD from three independent experiments. p-Values were determined using the unpaired t-test with Welch's correction.*

PIT of cancer were preferentially coupled *via* ester linkers to lysines (11), which, however, are uniformly distributed within an antibody molecule. Since all cysteines within an antibody molecule form inter- or intra-chain disulfide bridges for stabilization, the integration of free cysteines allows the specific coupling of our dye. With our method homogeneous antibody-dye conjugates can be generated. Moreover, the dye uptake can be controlled by the number and positions of cysteines incorporated without impairing affinity and specificity of the antibody.

PIT of PSMA expressing PC cells with our mAb-conjugate resulted in an effective and specific reduction of cell viability, while the free dye did not induce cytotoxicity. Our hypothesis is that the hydrophilic dye alone cannot enter the cells, but requires the antibody in order to be effectively delivered to and taken up by the cells. Further experiments will be carried out to elucidate the molecular cell death mechanisms of the dye WB692-CB1. In PIT experiments with the silicon phthalocyanine dye IR700, it was found that upon irradiation, the water-soluble axial ligands were cleaved from the silicon core domain and replaced by hydroxyl groups (OH). This change caused the dye molecules to stack closely together, leading to aggregation of the conjugates and subsequent damage to the cell membranes (12).

PIT with our Fab-conjugate resulted in lower cytotoxicity compared to the mAb-conjugate. This can be attributed to the approximately 12-fold reduced monovalent binding affinity and the approximately 4-fold lower dye uptake of the Fab-conjugate. In contrast to the mAb, coupling of WB692-CB1 led to an approximately 22-fold reduced binding affinity of the Fab fragment. This could be due to steric inhibition of the Fab fragment by WB692-CB1 coupled to the cysteine at position 118. As a possible solution, a cysteine could be inserted at another amino acid position of the C_H1 domain in order to obtain more distance to the paratope. 3D modeling could be used in the future to identify more suitable amino acids that could be mutated for dye coupling without affecting affinity. Despite lower efficacy compared to conjugates with full antibodies, Fab-conjugates could be advantageous for future clinical use of the PIT. A

shorter half-life and an increased tumor penetration could lead to a higher tumor uptake and to reduced time intervals between injection of the conjugate and irradiation (13, 14).

Conclusion

Taken together, our new conjugates h3/F11$^{A118C/D265C}$-WB692-CB1 and Fab^{A118C}-WB692-CB1 are promising candidates for the PIT of PC. The implementation of PIT during RP with our conjugates could lead to the complete removal of tumor cells in the surgical area and avoidance of local recurrence. This would spare stressful follow-up treatments and enable a higher QoL and a longer OS of patients with PC.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

PW, RB: conceptualization; SW, NG, JS, SSS, SL, IW: data curation; SW, NG, JS, SSS, SL, IW: formal analysis; RB, PW: funding acquisition; SW, NG, JS, SSS, SL, IW, RB, PW: investigation; SW, NG, PW, IW: methodology; RB, PW: project administration; CG, RB, PW: resources; RB, PW, IW: supervision; SW, NG, JS, SSS, JS, RB, PW, IW: validation; SW, PW, IW: writing – original draft; SW, NG, JS, SSS, SL, CG, RB: writing – review & editing.

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