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Photoimmunotherapy of HER2-expressing Breast Cancer Cells

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Abstract. *Background/Aim: Breast cancer (BC) is the most common malignant disease worldwide. Localized stages of BC can be successfully treated by surgery. However, local recurrence occurs in about 4-10% of patients, requiring systemic treatments that impair the patients' quality of life and shortens life expectancy. Therefore, new therapeutic options are needed, which can be used intraoperatively and contribute to the complete removal of residual tumor cells in the surgical area. In the present study, we describe a cysteine-modified variant of the anti-HER2 antibody trastuzumab, that was coupled to the silicon phthalocyanine photosensitizer dye WB692-CB1 for the photoimmunotherapy (PIT) of BC. Materials and Methods: The cysteine modified trastuzumab variant was cloned and expressed in Expi293F cells. After purification via immobilized affinity chromatography, the antibody was coupled to the dye. Cell binding of the antibody and the antibody dye conjugate was measured by flow cytometry. After incubation of BC cells with the conjugate and activation of the dye by irradiation with red light, cell viability was determined. Results: The antibody and the conjugate showed specific binding to HER2-expressing BC cells. Treatment of the HER2^{high} BC cell line SK-BR-3 with the conjugate followed by irradiation with a red light dose of 32 J/cm^2 led to complete cell killing within 24 h. Conclusion: Our novel antibody dye conjugate represents a promising candidate for intraoperative treatment of localized BC, aiming to eliminate residual tumor cells in the surgical area and potentially reduce local recurrence, thereby improving recovery prospects for BC patients.*

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Breast cancer (BC) is the most common diagnosed tumor entity worldwide. Almost 685,000 people died from the disease in 2020, making BC the fifth most common cause of cancer deaths (1). In localized stages of BC, the tumor is preferably resected by breast-conserving surgery or mastectomy (2). In the case of rapidly growing tumors, systemic or targeted neoadjuvant therapy can be applied to reduce the tumor mass preoperatively. Surgery is usually followed by radiotherapy to reduce the risk of recurrence and metastasis. Nevertheless, there is local recurrence in 4.4-10.3% of patients 5 years after surgery (3). In advanced stages, chemotherapy is the mainstay of treatment, which can be combined with targeted treatment depending on the molecular pathological subtype (4). In metastatic BC, therapy focus on prolongation of survival and maintenance of the patients' quality of life (5).

An important antigen for new targeted treatments of BC is HER2 (HER2/neu, ERB-B2), a member of the epidermal growth factor receptor family (6). About 20-30% of tumors in patients with localized BC show HER2 overexpression (7, 8). They are characterized by an aggressive growth and increased tendency to spread and are associated with poor prognosis in terms of recurrence and life expectancy (9-11). The monoclonal anti-HER2 antibodies trastuzumab and pertuzumab along with corresponding antibody drug conjugates, like trastuzumab emtansine and trastuzumab deruxtecan, are used for treatment of advanced BC (12).

In the present study, we describe the use of a cysteine-modified variant of trastuzumab, which was coupled to the

silicon phthalocyanine photosensitizer dye WB692-CB1 for the photoimmunotherapy (PIT) of BC. In PIT, the photosensitizer dyes are guided by highly selective antibodies to the tumor cells. After antigen binding and internalization of the conjugates, the dyes can be activated by irradiation with visible light and induce cell death (13).

Materials and Methods

Cell lines. The BC cell lines SK-BR-3 and MCF-7 (ATCC, Manassas, VA, USA) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) medium (Invitrogen, Karlsruhe, Germany) and the control cell line CHO (Invitrogen) in F-12 Nutrient Mixture Medium (Invitrogen), all supplemented with 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.

Generation of anti-HER2 antibodies. The anti-HER2 antibody trastuzumab (TRA) and a variant thereof containing two cysteine mutations at the amino acid positions T120 and D265 (EU numbering) in the heavy chain ($TRA^{T120C/D265C}$, Figure 1A and B) were generated as follows: constant domains of the heavy chain including the hinge region of a human IgG1 antibody with or without the cysteine mutations were synthesized (GeneArt, Invitrogen, Regensburg, Germany) and cloned into the expression vector pCSEH1c (14) *via NheI*/*XbaI* restriction sites (Figure 1C). The variable domain of the heavy chain (V_H) of trastuzumab was also synthesized and cloned into the vectors with the constant domains *via BssHII*/*NheI* restriction sites. The variable domain of the light chain (V_L) of trastuzumab was synthesized and cloned into the vector pCSL3k containing the constant domain of the light chain (C_L) of a human IgG1 antibody *via AgeI*/*XbaI* restriction sites (Figure 1C) (14). For transfection, 5×10^5 Expi293F cells (Thermo Fisher Scientific) were cultivated in FreeStyle F17 Medium + 8 mM L-Glutamin + 0.01% Pluronic F-68 (Pan Biotech, Aidenbach, Germany) in a 250 ml polycarbonate Erlenmeyer flask and incubated for 48 h at 37˚C, 5% CO_2 on a shaker (100 rpm). Then, the heavy and light chain vectors were mixed at a 1:1 ratio and a concentration of 1 μg/ml DNA in 1.25 ml FreeStyle F17 Expression Medium (Thermo Fisher Scientific, Paisley, UK). Simultaneously, 5 μg fully hydrolyzed polyamines (PEI Max, Polysciences Inc., Hirschberg an der Bergstrasse, Germany) were diluted in 1.25 ml FreeStyle F17 Expression Medium (Thermo Fisher Scientific). The DNA and PEI preparations were then mixed together and incubated at RT for 30 min to initiate complex formation. Then the DNA/PEI complex was added, and the cells were incubated again for 48 h in the incubator. For antibody production, 25 ml HyClone SFM4Transfx-293 Medium (Cytiva, Freiburg, Germany) + 8 mM L-Glutamin (Pan Biotech, Aidenbach, Germany) + 20% HyClone Cell Boost 6 supplement were added to the cells. Cell number and viability of the EXPI293F cells were monitored by LUNA cell counter (Thermo Fisher Scientific). Seven days after transfection, cell medium was centrifuged (1,500×*g*, 15 min), 1:1 diluted with PBS (pH 7.0) and sterile-filtered (0.22 μm, Millex GV Merck, Darmstadt, Germany). Purification of the antibodies TRA and $TRA^{T120C/D265C}$ by protein G affinity chromatography (Cytiva) was done according to the manufacturer's instructions. Purified antibodies were dialyzed against PBS (pH 7.4).

Generation of the antibody–dye conjugate. The cysteine modified antibody $TRA^{T120C/D265C}$ was dissolved at a concentration of 1 mg/ml in PBS, 1 mM EDTA (pH 7.4) and reduced with 40-fold equimolar Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) for 3 h and 37˚C on a shaker. After overnight dialysis against PBS, 1mM EDTA, pH 7.4, at 4˚C, the antibody was re-oxidized by use of dehydroascorbic acid (Sigma-Aldrich) for 4 h on a shaker. For dye conjugation, 1 μg/μl WB692-CB1 in DMSO was added at a molar ratio of 10:1 to the re-oxidized antibody and incubated for 15 min on a shaker in the dark. To remove free dye molecules, the antibody solution was incubated with N-acetyl-L-cysteine for 15 min on a shaker, dialyzed against PBS (pH 7.4) at 4˚C, overnight, and again purified by protein G affinity chromatography. After dialysis against PBS (pH 7.4) the concentration of the antibody dye conjugate $TRA^{T120C/D265C}$-WB692-CB1 was determined by Pierce BCA™ Protein-Assay (Thermo Fisher Scientific). Measurement of excitation and emission spectra of the conjugate solved in PBS was done with help of a microplate reader (FLUOstar OPTIMA, BMG LABTECH, Ortenberg, Germany).

SDS-PAGE. The antibodies TRA and $TRA^{T120C/D265C}$ and the antibody dye conjugate $TRA^{T120C/D265C}$-WB692-CB1 were analyzed by SDS-PAGE under non-reducing or reducing conditions according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and viewed under white or red light (λ=680 nm) using an IVIS 200 Imaging System (Spectral Instruments, Tuscon, AZ, USA).

Western blot analysis. Cells were lyzed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% sodium desoxycholate, 0.05% SDS, 1% Igepal. Protein concentrations of the lysates were determined with help of the Quick Bradford Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After blotting of 100 μg lysates per lane onto nitrocellulose membranes, HER2 was detected using the rabbit anti-human HER2 monoclonal antibody D8F12 (Cell Signaling Technology Europe, Leiden, the Netherlands) and horse radish peroxidase (HRP)-labeled polyclonal goat anti-rabbit IgG (Dako, Hamburg, Germany). Beta-actin was detected as loading control using the HRP-labeled rabbit anti-human β-actin antibody 13E5 (Cell Signaling). For the detection of the anti-HER2 antibodies, the HRP labeled polyclonal rabbit anti-Human IgG (Dako) was used. Blots were developed using an enhanced chemiluminescence (ECL) system. Protein bands were analyzed using an INTAS Chemo Star Imager (INTAS Science Imaging Instruments, Göttingen, Germany).

Flow cytometry. Binding of the antibodies and the conjugate was examined by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany) using goat-anti-human Ig(H+L)-RPE (Southern Biotech, Birmingham, AL, USA) as detection antibody as described (15). Dissociation constants (K_D) were defined as the antibody or conjugate concentrations leading to half-maximal specific cell binding and calculated using the software GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA).

Photoimmunotherapy of BC cells. BC cells were seeded at a concentration of 2.5×10^5 cells in 2 ml medium per 35 mm^2 petri-dishes overnight. After addition of 10 μg/ml $TRA^{T120C/D265C}$-WB692-CB1, $TRA^{T120C/D265C}$ or an equimolar concentration of free WB692-CB1 dye for 24 h, cell culture medium was exchanged and cells were irradiated with different doses of red light (690±10 nm) from a LED lamp (L690-66-60; Marubeni America Co., Dinslaken, Germany). 24 h after irradiation, cells were trypsinized, stained with Erythrosin B (Logos Biosystems, Gyeonggi-do, Republic of Korea) and counted using a Neubauer Counting

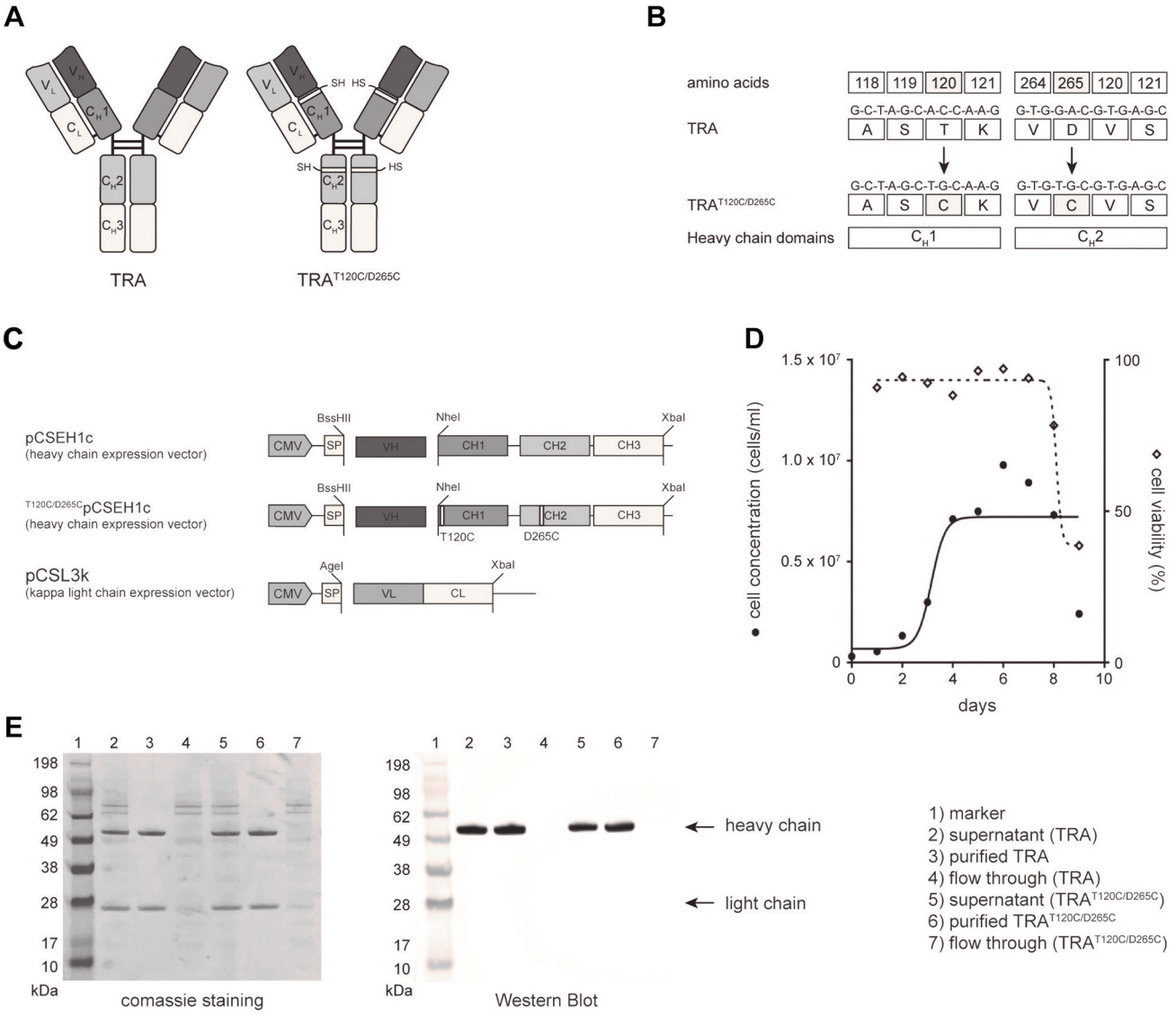

Figure 1. *Cloning and expression of the recombinant cysteine-modified anti-HER2 antibody TRA$^{T120C/D265C}$. (A) Schematic illustration of the antibody TRA and the antibody TRA$^{T120C/D265C}$ presenting free SH groups from the modified cysteines for dye coupling. (B) Cysteine mutations T120C and D265C in the C_H1 and C_H2 regions of the heavy chain of TRA$^{T120C/D265C}$ (EU numbering). (C) Schematic representation of the hIgG1 heavy chains of the antibodies TRA and TRA$^{T120C/D265C}$ in the eukaryotic expression vector pCSEH1c and the kappa light chain of both antibodies in the vector pCLS3k (14). (D) Growth and viability of Expi293F cells producing the antibodies. (E) Detection of TRA and TRA$^{T120C/D265C}$ in SDS gel and western blot after purification under reducing conditions using HRP labeled polyclonal rabbit anti-human IgG as detection antibody.*

Chamber. Cell viability was calculated as percentage of living cells normalized to the untreated control. 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 heavy and light chains of the anti-HER2 antibodies TRA and TRA$^{T120C/D265C}$ were cloned into the vectors pCSEH1c and pCSL3k (14), respectively (Figure 1A-C) and transduced into Expi293F cells for eukaryotic expression. As shown in Figure 1D, the Expi293F cells reached highest concentration and viability 6-8 days after seeding followed by rapid decrease in viability until day 9. We, therefore, decided to harvest the cells on day 7 and to purify the supernatants containing the antibodies by protein G affinity chromatography (Figure 1E). An average yield between 62 and 144 mg/l cell culture medium was reached for both antibodies after purification. In the next step, we coupled the silicon phthalocyanine photosensitizer dye WB692-CB1 (Figure 2A) to the free cysteines of

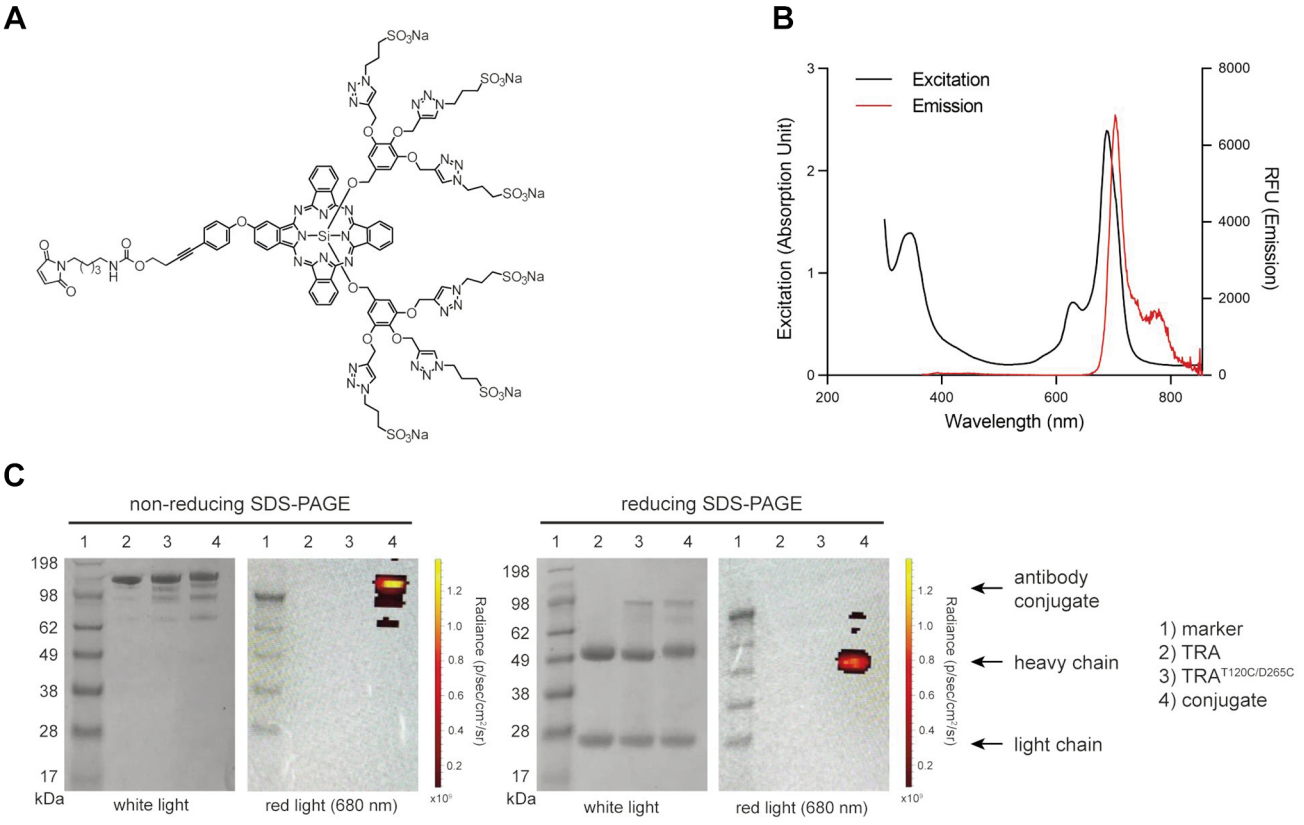

Figure 2. *Generation and characterization of the antibody dye conjugate TRA$^{T120C/D265C}$-WB692-CB1. (A) Structure of the silicon phthalocyanine photosensitizer dye WB692-CB1 with the maleimide linker for coupling to sulfhydryl groups from cysteines. (B) Absorption/emission spectra of the antibody dye conjugate TRA$^{T120C/D265C}$-WB692-CB1. (C) Antibody TRA$^{T120C/D265C}$ and the antibody dye conjugate TRA$^{T120C/D265C}$-WB692-CB1 in SDS gels analyzed under reducing and non-reducing conditions and detected under white and red light (λ=680 nm).*

TRA$^{T120C/D265C}$. Measurement of the absorption/emission spectra revealed a λ Ex_{max} of 689 nm and a λ Em_{max} of 704 nm for the conjugate TRA$^{T120C/D265C}$-WB692-CB1 (Figure 2B). This means that coupling to the antibody shifted the excitation maximum of the dye from original 692 nm by 3 nm to 689 nm, while the emission maximum remained the same at 704 nm. SDS PAGE analysis under non-reducing and reducing conditions and under white and red light proved that the dye was successfully coupled to the cysteine modified heavy chains of the antibody (Figure 2C).

We identified SK-BR-3 as a HER2^{high} expressing BC cell line and MCF-7 as a HER2^{low} expressing BC cell line by western blot analysis. The control cell line CHO was found to be HER2 negative (Figure 3A). Accordingly, high binding of the conjugate TRA$^{T120C/D265C}$-WB692-CB1 to SK-BR-3 cells and low binding to the MCF-7 cells was detected by flow cytometry (Figure 3B). PIT of SK-BR-3 cells, using 10 μg/ml TRA$^{T120C/D265C}$-WB692-CB1 followed by irradiation with a red light dose of 32 J/cm^2, led to nearly complete cell killing. Twenty-four h after irradiation, only 1.11±0.47% of the cells survived the treatment (p=0.0035). Cells that were incubated with the uncoupled antibody TRA$^{T120C/D265C}$ or with the free WB692-CB1 dye were not affected by irradiation. Moreover, cells that were incubated with the conjugate and were not irradiated also remained alive (Figure 3C). In contrast to the HER2^{high} SK-BR-3 cells, the HER2^{low} MCF-7 cells and the HER2 negative CHO cells were not damaged by treatment (Figure 3D and E). To investigate the dependence of cytotoxicity on the applied light dose, we incubated the SK-BR-3 cells with TRA$^{T120C/D265C}$-WB692-CB1 and irradiated them with different light doses ranging from 4 to 32 J/cm^2. As shown in Figure 3F, we found a significant increase in cytotoxicity after PIT correlating with increasing light doses.

Discussion

Photoactivatable fluorescent dyes are used for the photodynamic therapy (PDT) of chronic inflammation, drug-resistant bacterial infections or oncological diseases (16). In PDT of cancer, the dyes are administered topically or systemically and irradiated with light to induce cancer cell death. The advantages of PDT over conventional cancer

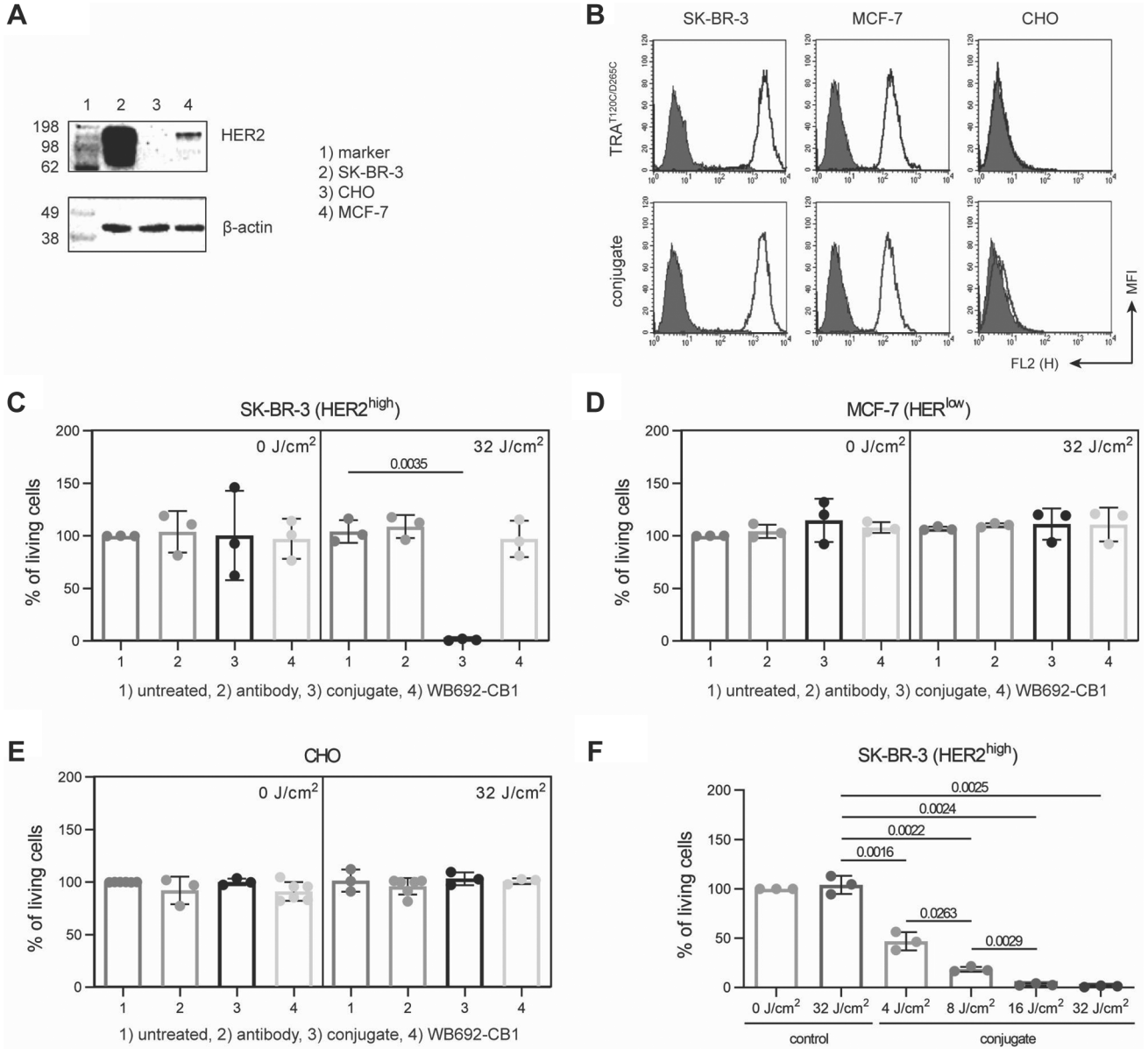

Figure 3. *Photoimmunotherapy of BC cells with the antibody dye conjugate TRA$^{T120C/D265C}$-WB692-CB1. (A) HER2 expression of the target cell lines as shown by western blot. β-actin was used as loading control. (B) Binding of the antibodies TRA, TRA$^{T120C/D265C}$ and the antibody dye conjugate TRA$^{T120C/D265C}$-WB692-CB1 to the target cells as shown by flow cytometry using the detection antibody goat-anti-human Ig(H+L)-RPE. Grey shaded peaks of control cells incubated with the detection antibody alone. Viability of (C) SK-BR-3 cells, (D) MCF-7 cells and (E) CHO cells after PIT. (F) Viability of SK-BR-3 cells after PIT with various light doses. Mean±SD values of 3-6 independent experiments. Statistical significance was calculated using unpaired t-test with Welch's correction.*

therapies are the low systemic toxicity of the dyes and the spatio-temporal control of cell death induction by use of light sources without ionizing radiation (17). Obstacles of the dyes are their hydrophilic properties, which hinders them to diffuse into the cells through the cell membrane, and their lack of tumor specificity, leading to damage of surrounding healthy tissue, that has absorbed the dye and was irradiated (18).

The silicon phthalocyanine dye WB692-CB1 has a maleimide linker to be specifically coupled to sulfhydryl groups, for example of free cysteines. Since all cysteines naturally present in an antibody form intra- or inter-chain disulfide bridges, mutation of suitable amino acids to cysteines can be used for dye coupling. Our dye is therefore advantageous to previous dyes, which are coupled to ubiquitously distributed lysines in an antibody, so that

heterogeneous conjugate preparations are produced. Moreover, by incorporation of a various number of cysteines into antibodies the dye uptake can be controlled in future and the pharmacokinetics and effective properties of the conjugates can be influenced. For example, mutations of aspartic acid at position 265 were shown to reduce the affinity of antibodies to Fc gamma receptors (FcRys). FcRy interactions are responsible for immune responses such as antibody-dependent cytotoxicity and complement-dependent cytotoxicity (19). The mutation D265C could therefore lead to a reduction of unwanted immune responses in BC patients. Moreover, cysteines could be introduced into antibody domains that are responsible for interaction with the neonatal Fc receptor (FcRn) on endothelial cells (L309-Q311 of the C_H2 domain or L251-S254 and N434-H435 of the C_H3 domain) (167,176). FcRn interactions prevent intracellular lysosomal degradation of IgG antibodies taken up into endothelial cells and cause a relatively long serum half-life of approximately 21 days (20). Inhibition of FcRn interactions of our conjugates by cysteine mutations could evoke shorter serum half-lives that enable shorter intervals between conjugate application followed by intratumoral enrichment and light irradiation in the PIT of BC patients.

In this study, we established our new silicon phthalocyanine dye WB692-CB1 as a photosensitizer for the PIT of HER2 expressing BC cells by coupling it to a cysteine modified variant of the anti-HER2 antibody trastuzumab. Our conjugate $TRA^{T120C/D265C}$-WB692-CB1 is a promising candidate for future use in the PIT of BC, which is particularly suitable for intraoperative use against localized tumor stages to combat residual tumor cells in the surgical area. We demonstrated that HER2 negative cells remained protected from PIT with the conjugate and that neither the uncoupled antibody nor the free dye induced cytotoxicity even after light irradiation. Our photoimmunotherapeutic approach could, therefore, be a gentle alternative to the postoperative radiotherapy for the eradication of residual tumor cells in the surgical area, avoiding local recurrence and stressful follow-up treatments, and effectively contribute to the healing of BC patients.

Treatment of the $HER2^{high}$ BC cell line SK-BR-3 with $TRA^{T120C/D265C}$-WB692-CB1 followed by irradiation with a red light dose of 32 J/cm² led to complete cell killing within 24 hours, whereas the same treatment of $HER2^{low}$ MCF-7 cells did not result in cytotoxicity. This suggests, on the one hand, that effective PIT requires a certain HER2 antigen level on the cancer cells. On the other hand, it indicates that surrounding normal cells with naturally occurring low HER2 expression might remain unaffected by PIT, resulting in a corresponding therapeutic window. In addition, future experiments will reveal whether incubation with higher conjugate concentrations or irradiation with higher light doses can also trigger death of $HER2^{low}$ BC cells.

The cytotoxicity mechanisms of WB692-CB1 are still unknown and have to be examined in further studies. Experiments with conjugates comprising different antibodies and the phthalocyanine dye IR700 revealed a conformational change of the antigen-bound conjugate triggered by a photochemical axial ligand release reaction after irradiation. This was followed by cell membrane rupture and cellular influx of ions, ultimately leading to necrotic cell death (21). Other studies demonstrated a connection between PIT and an induction of oxidative singlet oxygen species, which led to lipid peroxidation and cell death (22).

PIT of localized BC with our trastuzumab-WB692-CB1 conjugate could be applied in tumors that were characterized as $HER2^{high}$ after immunohistochemical examination, *e.g.* in biopsies. In order to increase the efficacy of PIT, it is conceivable to use multiple conjugates that are directed against different target antigens in BC. For example, conjugates against HER2 could be combined with conjugates directed against VEGF or TROP-2, which were also identified to be overexpressed in BC and which can be targeted *e.g.* by the clinically approved antibodies bevacizumab and sacituzumab, respectively (23, 24).

Conclusion

Taken together, we found a highly specific and light dose dependent cytotoxicity of our new antibody dye conjugate $TRA^{T120C/D265C}$-WB692-CB1 in $HER2^{high}$ BC cells, which makes it a promising candidate for the future clinical application in the PIT of BC.

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Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

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

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