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Article

Exploring EGFR, Nectin-4, and TROP-2 as Therapeutic Targets for Bladder Cancer Photoimmunotherapy

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Abstract

Background: Non-muscle invasive bladder cancer (NMIBC) has limited therapeutic options and high recurrence rates. Photoimmunotherapy (PIT) enables targeted tumor ablation using antibody–photosensitizer conjugates and light activation. We evaluated EGFR, Nectin-4, and TROP-2 as PIT targets using cysteine-modified antibodies conjugated to the photosensitizer WB692-CB2. **Methods:** Antibodies derived from Cetuximab (Cmb, anti-EGFR), Enfortumab (Enf, anti-Nectin-4), and Sacituzumab (Sac, anti-TROP-2) were engineered with T120C and D265C mutations in the heavy chains for site-specific dye conjugation. Binding and light-induced cytotoxicity of the conjugates, alone or in combination, were assessed in BC cells by flow cytometry and viability assays following irradiation. **Results:** Cysteine-modified antibodies were produced as intact IgG molecules and efficiently conjugated with WB692-CB2 without loss of antigen specificity. Sac^{T120C/D265C}-WB692-CB2 showed the highest target binding and achieved near-complete cell killing at a red light dose of 32 J/cm². Cmb^{T120C/D265C}-WB692-CB2 required a fourfold higher light dose for comparable efficacy, while Enf^{T120C/D265C}-WB692-CB2 showed lower potency. No cytotoxicity was observed in antigen-negative cells. Combined treatment enhanced cytotoxicity, indicating additive phototherapeutic effects. **Conclusions:** Sac^{T120C/D265C}-WB692-CB2 represents a highly effective PIT conjugate for NMIBC. Our findings support PIT as a promising, targeted, and minimally invasive therapeutic option for BC, potentially adaptable for combinatorial or personalized strategies.

Keywords: EGFR; Nectin-4; TROP-2; photoimmunotherapy; antibodies; photosensitizer dye; WB692-CB2; bladder cancer

1. Introduction

Bladder cancer (BC) is one of the most common malignancies, with more than 610,000 new cases and 220,000 deaths annually. The disease ranks as the sixth most common cancer and the ninth leading cause of cancer death worldwide with highest incidence rates in Europe [1].

At diagnosis, about 70% of tumors are still confined to the urothelium or lamina propria and have not invaded the muscle layer [1]. For non-muscle-invasive BC (NMIBC), transurethral resection followed by intravesical chemotherapy with mitomycin C or gemcitabine is the standard therapy. Patients with intermediate, high and very high risk might also receive Bacillus Calmette-Guérin (BCG) immunotherapy to lower the likelihood of disease progression [2]. However, recurrence rates remain high, affecting about 31-78% of patients depending on risk group within 5 years after treatment [3]. Regular cystoscopic follow-up is therefore required. Progression to muscle-invasive BC

(MIBC) necessitates radical treatment such as cystectomy with chemotherapy or radio-chemotherapy [4]. In metastatic disease, prognosis for BC patients is poor with median survival of only a few months [5]. Recent advances in the treatment of BC include the use of immune checkpoint inhibitors (ICI) [6] and the application of antibody drug conjugates (ADC) such as enfortumab vedotin [7] and sacituzumab govitecan [8]. Despite these developments, clinical outcomes in BC remain unsatisfactory. Novel targeted therapies are needed to eradicate residual tumor cells in localized disease, to reduce recurrence, and to improve both survival and quality of life.

Photoimmunotherapy (PIT) represents a highly specific approach within the field of targeted photodynamic therapy (PDT). In this strategy, tumor-specific monoclonal antibodies, antibody fragments, or ligands are covalently coupled to photosensitizers (PS), thereby enabling preferential accumulation of the conjugates in malignant tissues through recognition of cell-surface antigens. Upon adequate tumor localization, the PS moiety can be selectively activated by radiation with red or near-infrared light, which penetrates tissue effectively and is non-ionizing in nature. Activation of the PS leads to the formation of reactive oxygen species (ROS), including singlet oxygen, peroxides, and hydroxyl radicals via photochemical reactions. ROS induce oxidative stress, leading to damage of cellular organelles and biomolecules, followed by the release of neoantigens and damage-associated molecular patterns (DAMPs), ultimately promoting immunogenic cell death [9]. PIT is particularly suitable for tumors that both (i) express antigens amenable to antibody targeting and (ii) are accessible to light delivery, either externally or via interstitial fiber-optic approaches. Compared to conventional chemotherapy or radiotherapy, PIT provides spatiotemporal precision and reduced systemic toxicity, while retaining the immunological advantages of PDT [10].

For the PIT of NMIBC, cell surface antigens like the epidermal growth factor receptor (EGFR), Nectin cell adhesion molecule 4 (Nectin-4) and the Trophoblast cell surface antigen-2 (TROP-2) are conceivable targets.

Overexpression of EGFR, a tyrosine kinase of the ErbB receptor family, correlates with tumor grade, muscle invasion and recurrence of BC and therefore serves as a prognostic marker. Based on immunohistological studies, EGFR is present in about 12-40% of NMIBC patients [11,12].

The cell adhesion molecule Nectin-4 is particularly overexpressed in various solid tumors, including urothelial cancer [13,14]. It promotes tumor angiogenesis, cell growth, proliferation, migration and epithelial-mesenchymal transition (EMT) and was found to be heterogeneously expressed in approximately 87-90% of patients with NMIBC [15–17]. Nectin-4 serves as target of the ADC enfortumab vedotin (EV), which is indicated as first-line therapy in combination with pembrolizumab for patients not suitable for platinum chemotherapy or for the treatment of patients with locally advanced or metastatic urothelial cancer, who have previously received ICI and a platinum-containing chemotherapy [18].

TROP-2 is a type I transmembrane glycoprotein consisting of 323 amino acids, serves as a calcium signal transducer and contributes to proliferation, adhesion, Epithelial-to-Mesenchymal Transition and death of tumor cells [13,19,20]. In an immunohistological panel of 102 patients with NMIBC, TROP-2 expression was detected in all samples and high expression of TROP-2 was significantly associated with tumor grade ($P=0.001$), stage ($P<0.001$), and recurrence ($P=0.03$) [21]. TROP-2 antigen is the target of the ADC sacituzumab govitecan, which is approved for the treatment of locally advanced or metastatic urothelial cancer in patients previously treated with platinum-based chemotherapy and ICI [22].

We have recently published the novel silicon phthalocyanine dye WB692-CB2 (Abs._{max} 692 nm / Em._{max} 703 nm), which is the first light activatable PS that can be directly conjugated to cysteines via a maleimide linker. Following incubation of prostate cancer cells with a conjugate composed of a cysteine-modified antibody directed against the prostate-specific membrane antigen (PSMA) and WB692-CB2, subsequent red-light irradiation effectively induced pyroptosis as main cell death mechanism [9]. In the present study, we established a PIT strategy against BC cells employing WB692-CB2-conjugated antibodies directed against EGFR, Nectin-4 and TROP-2.

2. Results

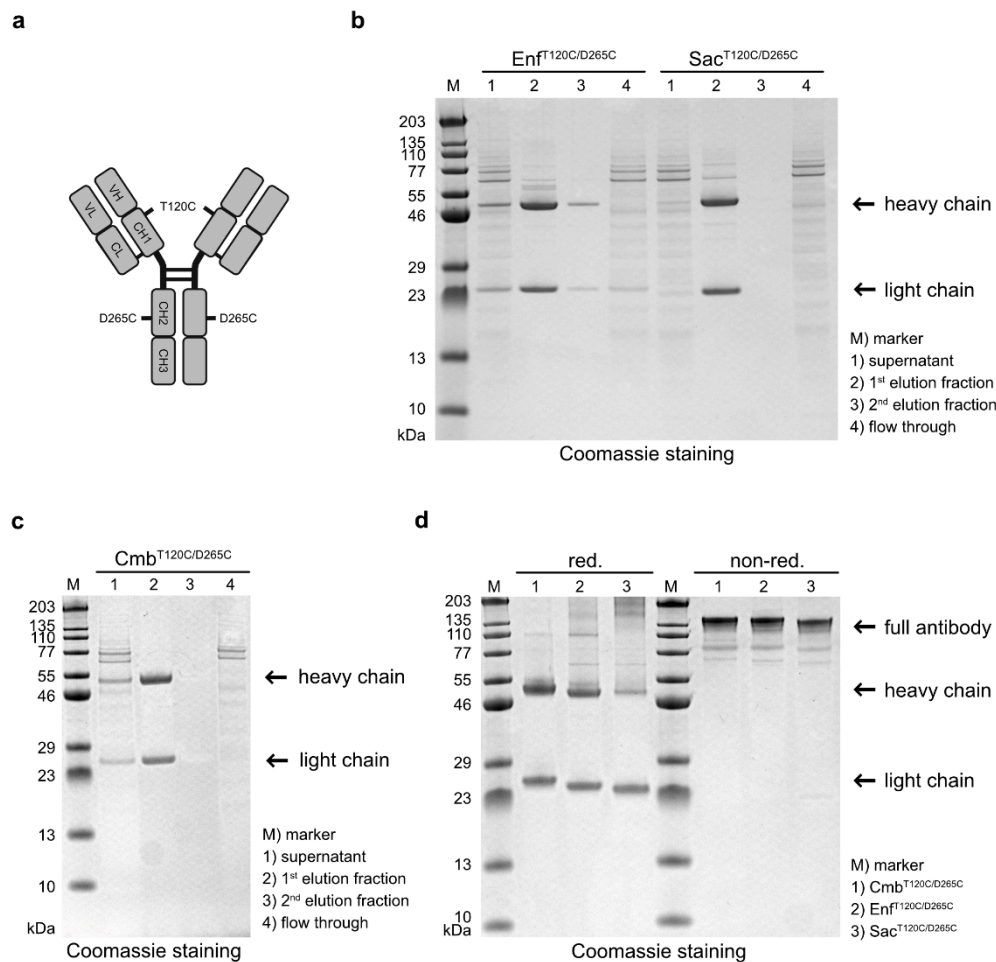


Figure 1. (a) Graphical representation of the antibodies carrying cysteine mutations T120C and D265C in the heavy chains, enabling coupling of the photosensitizer dye WB692-CB2. (b) SDS-PAGE analysis of the antibodies Enf^{T120C/D265C} and Sac^{T120C/D265C} after purification from cell culture supernatants by Protein G affinity chromatography. (c) SDS-PAGE analysis of the antibody Cmb^{T120C/D265C} after purification from cell culture supernatant by Protein G affinity chromatography. (d) SDS-PAGE of the antibodies after purification under reducing and non-reducing conditions.

Antibody variants targeting EGFR, Nectin-4 and TROP-2 were generated by cloning the VH and VL domains of Cetuximab (Cmb), Enfortumab (Enf) and Sacituzumab (Sac), respectively, into human hIgG1 heavy- and light chain expression vectors carrying cysteine mutations at positions T120 and D265 in the heavy chain (Figure 1a). After expression in Expi293T cells and purification by affinity chromatography, the cysteine-modified antibodies Cmb^{T120C/D265C}, Enf^{T120C/D265C} and Sac^{T120C/D265C} were analyzed by SDS-PAGE. All antibodies were detected in the first elution fraction (Figure 1b,c). Under reducing conditions, the expected ~ 49-50 kDa heavy and ~ 23 kDa light chains were observed, whereas under non-reducing conditions, intact antibodies with a molecular weight of ~ 145 kDa were detected (Figure 1d). This proved that all antibody variants were correctly assembled as full-length IgG molecules in our expression system.

In the next step, the PS dye WB692-CB2 was coupled to the cysteine-modified antibodies via a maleimide linker (Figure 2a). The insertion of two cysteine mutations per heavy chain theoretically allows the coupling of up to four dye molecules per antibody. Using our established coupling protocol, in which ten dye molecules were added per antibody molecule, we achieved dye-to-antibody ratios of 3.05, 3.08 and 3.13 for Cmb^{T120C/D265C}-WB692-CB2, Enf^{T120C/D265C}-WB692-CB2 and

Sac^{T120T/265C}-WB692-CB2, respectively. Analysis of the conjugates under red light (680 ± 10 nm) revealed fluorescence signals corresponding to the heavy chains (Figure 2b), confirming the successful coupling of the dye to the cysteine mutations.

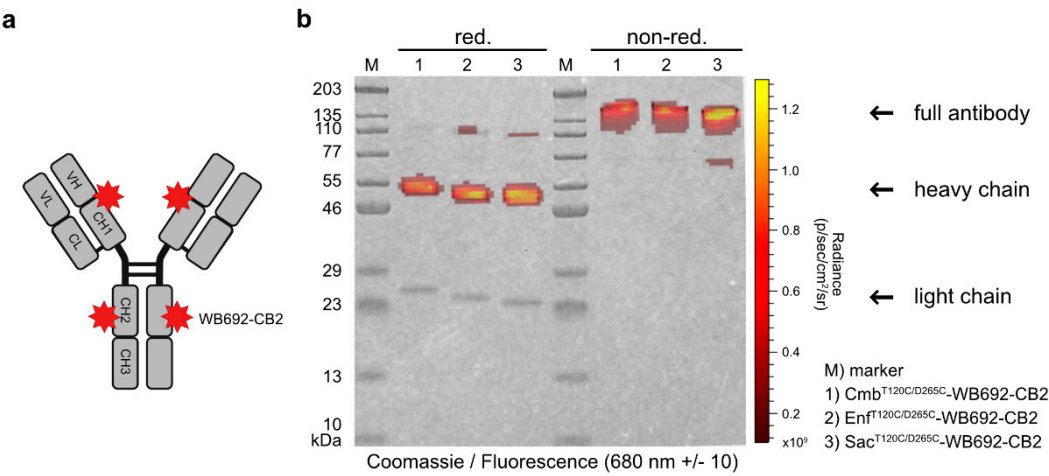


Figure 2. (a) Graphical representation of the antibody dye conjugates carrying cysteine coupled WB692-CB2 dyes. (b) Overlay of Coomassie-stained SDS-PAGE gels of the conjugates under reducing and non-reducing conditions, visualized under white and red light (680 ± 10 nm).

The binding properties of the antibodies and their conjugates were evaluated on RT4 and RT112 BC cells. Western Blot analyses of cell lysates confirmed RT4 cells were EGFR^{high}, Nectin-4^{high} and TROP-2^{high}, whereas RT112 cells were characterized as EGFR^{high}, Nectin-4^{low} and TROP-2^{high}. The hamster cell line CHO served as a negative control and showed no detectable expression of any of the three antigens (Figure 3a). Flow cytometry revealed strong and specific binding of the antibodies and their corresponding conjugates to RT4 and RT112 cells, with the highest mean fluorescence intensity (MFI) values observed for Sac^{T120C/D265C}, followed by Cmb^{T120C/D265C} and Enf^{T120C/D265C}, and their respective conjugates (Figure 3b). No binding was detected in CHO control cells. This proved that the coupling of our dye WB692-CB2 did not impair the antigen-specific binding of the antibodies.

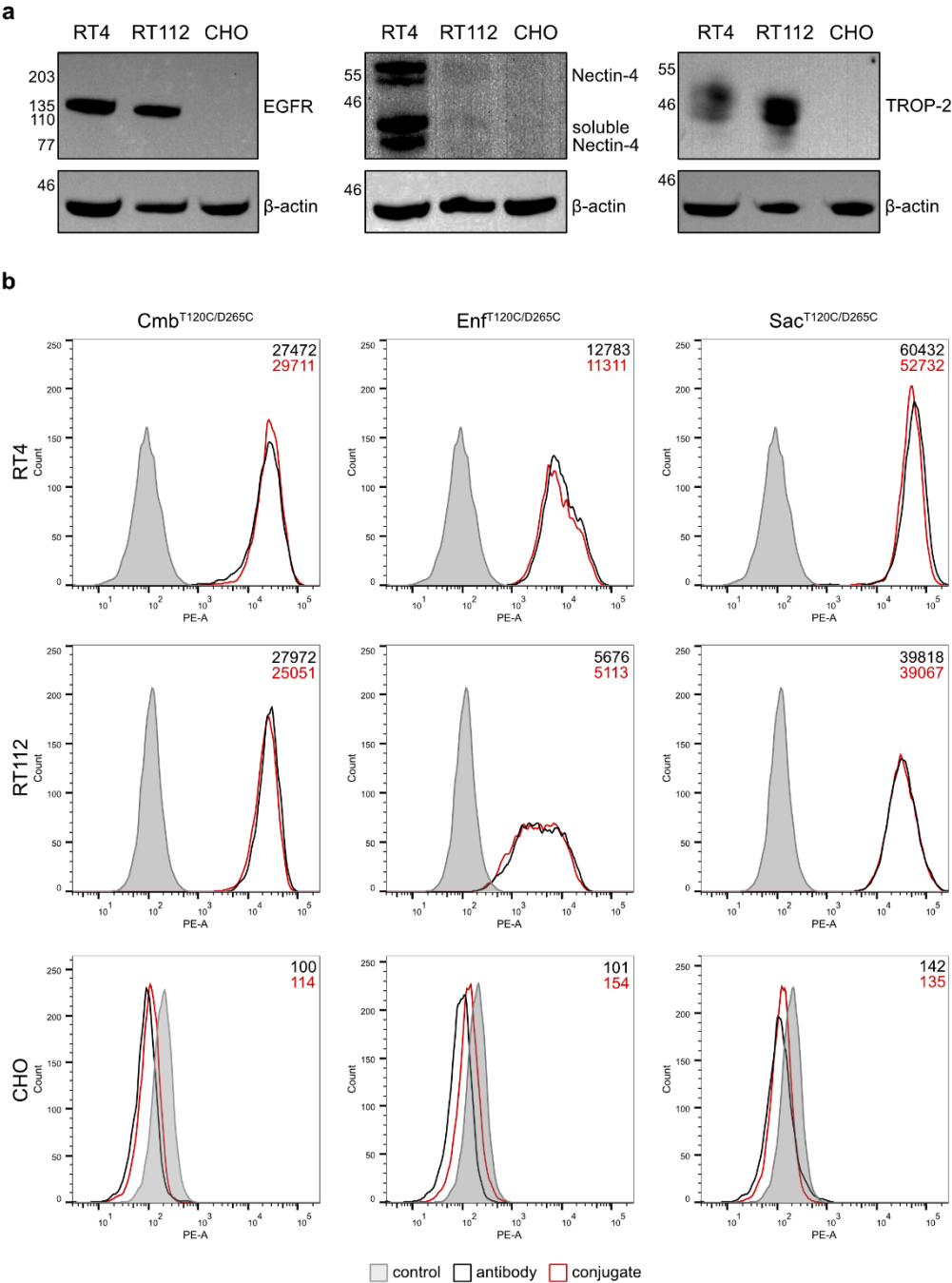


Figure 3. (a) Western blot analysis of EGFR, Nectin-4 and TROP-2 expression in the BC cell lines RT4 and RT112 and in the antigen-negative control cell line CHO. **(b)** Cell binding of the antibodies and conjugates to the target cells as demonstrated by flow cytometry. Binding of the isotype control antibody is shown in grey.

For PIT, BC cells were incubated with 10 μ g/ml of each conjugate and subsequently irradiated with varying doses of red light (690 nm, 0-128 J/cm²). All conjugates induced light dose-dependent cytotoxicity in the target cells (Sac^{T120C/D265C}-WB692-CB2 > Cmb^{T120C/D265C}-WB692-CB2 > Enf^{T120C/D265C}-WB692-CB2), consistent with their relative binding affinities. Treatment with Sac^{T120C/D265C}-WB692-CB2 significantly reduced RT4 cell viability to 6.3 $\pm$ 7.6% and completely eradicated RT112 cells at a light dose of 32 J/cm². Nearly complete ablation of BC cells was achieved with Cmb^{T120C/D265C}-WB692-CB2 at a dose of 128 J/cm², resulting in 2.1 $\pm$ 2.3% and 3.6 $\pm$ 1.5% viability for RT4 and RT112 cells, respectively. In contrast, Enf^{T120C/D265C}-WB692-CB2 exhibited the weakest phototoxic response, reducing RT4 cell viability to 13.1 $\pm$ 3.3% and RT112 cell viability to 74.2 $\pm$ 2.4% at 128 J/cm². No

cytotoxic effects were observed in the antigen-negative CHO control under any conditions, demonstrating the high specificity and safety of our PIT approach (Figure 4).

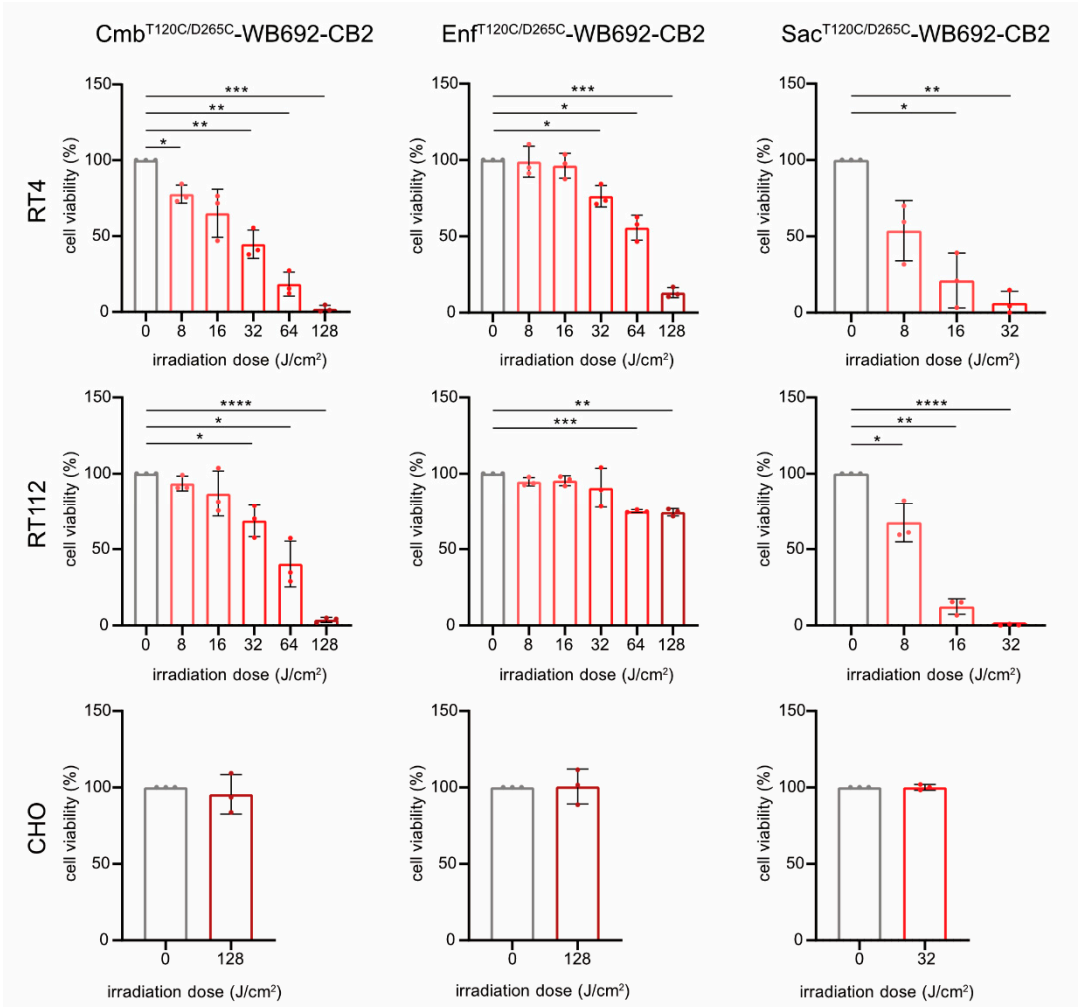


Figure 4. Cytotoxicity of the conjugates Cmb^{T120C/D265C}-WB692-CB2, Enf^{T120C/D265C}-WB692-CB2 and Sac^{T120C/D265C}-WB692-CB2 after irradiation with different light doses in RT4 and RT112 cells. CHO cells served as antigen-negative controls. Mean values $\pm$ SD of three independent biological experiments. Statistical analyses were performed using unpaired, parametric Student's *t*-tests with Welch's correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$).

The simultaneous application of multiple conjugates was investigated to assess potential additive cytotoxic effects. BC cells were co-incubated with the conjugates Cmb^{T120C/D265C}-WB692-CB2 and Enf^{T120C/D265C}-WB692-CB2 and subsequently irradiated with a light dose of 64 J/cm². Indeed, the combined treatment with both conjugates resulted in a markedly enhanced cytotoxic response compared with either conjugate alone, indicating additive phototoxic effects. In contrast, neither the unconjugated antibodies nor the free WB692-CB2 dye exhibited any cytotoxicity under the same experimental conditions, confirming that light-induced cell killing was strictly dependent on targeted photoactivation of the conjugates (Figure 5).

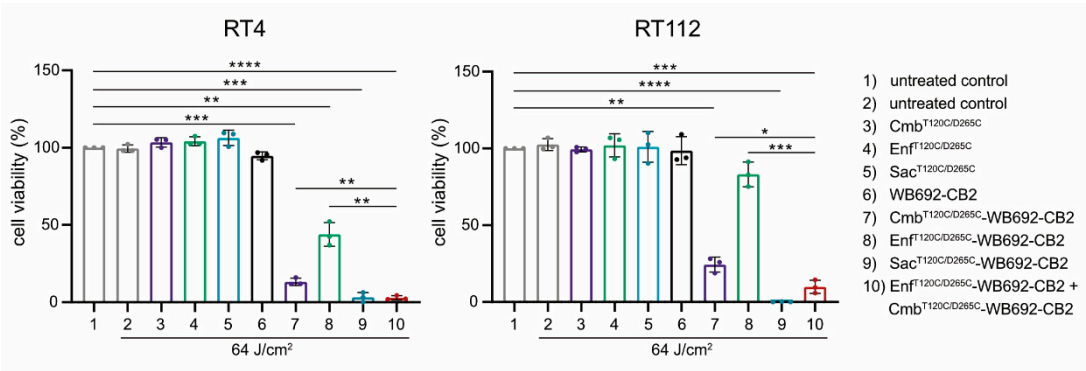


Figure 5. Cytotoxicity of the conjugates Cmb^{T120C/D265C}-WB692-CB2, Enf^{T120C/D265C}-WB692-CB2 and Sac^{T120T/265C}-WB692-CB2 alone and in combination after irradiation with a light dose of 64 J/cm² in RT4 and RT112 cells. Control samples were treated with the uncoupled antibodies or the free dye. Mean values ± SD of three independent biological experiments. Statistical analyses were performed using unpaired, parametric Student's *t*-tests with Welch's correction (**p* < 0.05, ***p* < 0.01, ****p* < 0.005, *****p* < 0.001).

Collectively, these results demonstrate that cysteine-engineered antibody dye conjugates targeting EGFR, Nectin-4, and TROP-2 retain their antigen-specificity and induce highly selective, light-dependent cytotoxicity in BC cells. Moreover, the observed additive effects upon combined application of different conjugates suggest an additive therapeutic potential that could enhance treatment efficacy and offer meaningful benefits for patients with heterogeneous tumor antigen expression.

3. Discussion

EGFR, Nectin-4 and TROP-2 represent valuable targets for the PIT of NMIBC, as they are highly expressed on the surface of BC cells and undergo internalization after antibody binding [12,14,20]. In this study, we demonstrate that the antigen-binding domains of Cetuximab, Enfortumab and Sacituzumab, originally employed as ADCs for the targeted delivery of cytotoxic agents to cancer cells [23,24], can also be applied to generate cysteine-modified antibodies as vehicles for intracellular transport of the photosensitizer dye WB692-CB2 in PIT. Consequently, the PIT approach against BC could be extended to other therapeutic antibodies, which are currently being tested in clinical trials in the form of ACDs directed against human epidermal growth factor receptor 2 (HER-2), tissue factor (TF) or epithelial cell adhesion molecule (EpCAM) [25].

Based on the MFI values gained from our flow cytometric analyses, we found highest binding of the anti-TROP-2 conjugate Sac^{T120C/D265C}-WB692-CB2 to RT4 and RT112 cells, followed by the anti-EGFR conjugate Cmb^{T120C/D265C}-WB692-CB2 and the anti-Nectin-4 conjugate Enf^{T120C/D265C}-WB692-CB2. Accordingly, the highest cytotoxicity after PIT was achieved with the conjugate Sac^{T120C/D265C}-WB692-CB2. At a conjugate concentration of 10 µg/ml and a light dose of 32 J/cm², almost complete elimination of the BC cells was observed. In contrast, Cmb^{T120C/D265C}-WB692-CB2 required a fourfold higher light intensity (128 J/cm²) to achieve comparable efficacy, while Enf^{T120C/D265C}-WB692-CB2 induced only limited cytotoxicity, reducing RT112 and RT4 cell viability by approximately 26% and 87% at the same dose, respectively. Overall, we identified Sac^{T120T/D265C}-WB692-CB2 as the most effective conjugate for PIT of BC cells, followed by Cmb^{T120C/D265C}-WB692-CB2 and Enf^{T120C/D265C}-WB692-CB2. Beyond differences in antigen expression, the observed variation in the cytotoxicity among the conjugates may be attributable to differences in antigen internalization kinetics or endolysosomal degradation. Further investigations are required to elucidate the relative contributions of these processes to PIT efficacy with our dye.

We have recently shown that PIT with WB692-CB2 induced pyroptosis as main cell death mechanism in prostate cancer cells [9]. Pyroptosis is a nonapoptotic form of programmed cell death characterized by inflammasome activation and caspase-dependent cleavage of gasdermin D, leading

to the formation of pores in the cell membrane and cell burst [26]. This causes the release of pro-inflammatory molecules that recruit lymphocytes and boost the immune system’s capacity to eliminate tumor cells. Induction of pyroptosis with antineoplastic agents is increasingly coming into focus to combat apoptosis-resistant tumor cells and to enhance the antitumor efficacy of ICI [27]. Our future investigations will show whether this is also the case in BC cells. If confirmed, PIT could not only eradicate tumor cells but also be used to prime the immune system of NMIBC patients against tumor cells that have already spread [28].

NMIBC can be multifocal and exhibits pronounced intra- and intertumoral antigen heterogeneity, arising from genetic mutations, altered molecular pathways, and the influence of the tumor microenvironment. This inherent heterogeneity influences disease progression, recurrence, and therapeutic outcome [17,29,30]. In clinical application of PIT, it could therefore be advantageous to target different tumor antigens simultaneously. We therefore treated the BC cells with Cmb^{T120C/D265C}-WB692-CB2 and Enf^{T120C/D265C}-WB692-CB2 and found additive cytotoxic effects. Our observations are in line with another study, where higher efficacy was reached with a combinatorial PIT using conjugates consisting of the anti-EGFR antibody panitumumab or the anti-HER-2 antibody trastuzumab and the lysine-coupling PS dye IR700 compared to monotherapies in BC cells [31]. In the context of personalized medicine, it may become feasible to access antigen expression profiles before and during PIT by biopsies and to tailor the administration of appropriate antibody dye conjugates accordingly. This could improve therapeutic outcome and reduce the risk of tumor recurrence.

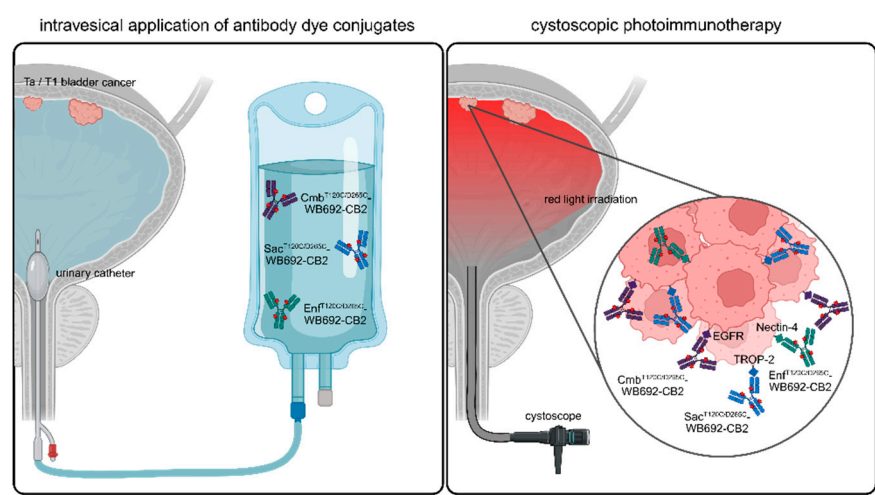


Figure 6. Graphical abstract illustrating the future clinical application of PIT using WB692-CB2 conjugates for the treatment of NMIBC. Following intravesical application of the antibody dye conjugates into the bladder, they can be activated by cystoscopic red light for the selective eradication of BC cells expressing EGFR, Nectin-4 and/or TROP-2. For personalized PIT, a pre-therapeutic determination of target antigen expression in biopsy samples may guide the selection of an optimal conjugate combination. Created in BioRender. Wolf, I (2025) <https://BioRender.com/sx866ld>.

In this study, we demonstrated that PIT represents a novel, targeted and gentle therapeutic option for NMIBC. It can be performed in future by instilling the bladder with respective antibody dye conjugates via a catheter, followed by light irradiation using a cystoscope (Figure 6). Due to its high specificity and the use of harmless light, PIT can be considered a gentle and effective alternative to conventional BCG treatment or chemotherapy.

4. Materials and Methods

4.1. Cell Lines

The EGFR-, Nectin-4- and TROP-2-positive cell lines RT4, derived from a patient with a recurrent well-differentiated transitional papillary tumor, and RT112, derived from a patient with a transitional cell carcinoma of the urinary bladder, were purchased from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Braunschweig, Germany). The negative control cell line CHO was obtained from Gibco (Invitrogen, Karlsruhe, Germany). RT4 cells were cultivated in EMEM medium (Cyton, Eppelheim, Germany), RT112 cells in RPMI1640 medium (Gibco), and CHO cells in F-12 Nutrient Mixture Medium (Gibco) at 37°C and 5% CO₂. All media were supplemented with 10% fetal calf serum (Sigma Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (100 U/ml, 100 mg/l, Sigma Aldrich).

4.2. Antibody Generation

For the generation of the heavy chains of the cysteine-modified antibodies Cetuximab (Cmb^{T120C/D265C}, www.kegg.jp: D03455), Enfortumab (Enf^{T120C/D265C}, www.kegg.jp: D11524) and Sacituzumab (Sac^{T120C/D265C}, www.kegg.jp: D10984), the variable heavy chain (V_H) domains and constant heavy chain (CH) domain genes of a hIgG1 antibody carrying the T120C and D265C (EU numbering) cysteine mutations were synthesized by Gene Art technology optimized for eukaryotic expression (Invitrogen, Regensburg, Germany). The variable light chains (V_L) domains of the three antibodies were synthesized in parallel. The heavy chains were cloned into the expression vector pCSEH1c, and the light chains into the expression vector pCSL3k containing a human IgG1 constant light chain (CL) domain, as described previously [9]. Transformation of the constructs was carried out in XL1-Blue MRF' supercompetent *E. coli* cells (Agilent Technologies, Waldbronn, Germany), and plasmid DNA was purified using the NucleoBond® Xtra Maxi Kit (Macherey-Nagel, Düren, Germany). All sequences were verified by Microsynth Seqlab (Göttingen, Germany).

Recombinant antibody expression was performed in EXPI293F cells (Thermo Fisher Scientific, Waltham, MA, USA) using established protocols (PMID: 39246837, Wolf et al., 2024). The antibodies were purified from cell culture supernatant by affinity chromatography. In brief, supernatant containing the antibody was 1:1 diluted in PBS (pH 7.0) and pumped over a Protein G column (Cytiva, Marlborough, MA, USA). The column was washed with PBS (pH 7.0), and the antibodies were eluted with 0.1 M glycine-HCl (pH 2.5) in two 1 ml fractions. After neutralization with 1 M Tris-HCl (pH 9.0), the antibodies were dialyzed against PBS (pH 7.4), and stored at -20 °C. Final antibody concentrations were determined using a NanoDrop Lite™-spectrophotometer (Thermo Fisher Scientific).

3. Generation of the Antibody-Dye Conjugates

The cysteine modified antibodies Cmb^{T120C/D265C}, Enf^{T120C/D265C} and Sac^{T120C/D265C} were diluted in PBS, 1mM EDTA (pH 7.4) and reduced with 40 equimolar Tris-(2-Carboxyethyl)phosphine, hydrochloride (TCEP, Carl Roth, Karlsruhe, Germany) for 3 h at 37 °C on a shaking platform. Overnight dialysis against PBS, 1mM EDTA (pH 7.4) at 4°C was followed by re-oxidation with 30 equimolar dehydroascorbic acid (dhAA, Sigma-Aldrich, St. Louis, USA) for 4 h at room temperature. For dye conjugation, 10-fold equimolar WB692-CB2 dye was added for 1h at room temperature on a shaker, protected from light. 25-fold equimolar N-acetyl-L-cysteine (Sigma-Aldrich) was added for 15 minutes to quench the reaction. For elimination of unbound dye, the antibody-dye-solutions were purified using Protein G affinity chromatography (Cytiva), followed by dialysis against PBS (7.0). To quantify the protein concentration, Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) was utilized. Dye to protein ratios were calculated as described [9].

4. SDS PAGE

The antibodies Cmb^{T120C/D265C}, Enf^{T120C/D265C} and Sac^{T120C/D265C}, as well as their corresponding antibody dye conjugates Cmb^{T120C/D265C}-WB692-CB2, Enf^{T120C/D265C}-WB692-CB2 and Sac^{T120C/D265C}-WB692-CB2, were analyzed by SDS-PAGE under reducing and non-reducing conditions. Protein bands were visualized by Coomassie staining (Protein Ark, Sheffield, UK) and fluorescence-based imaging ($\lambda = 680$ nm) using an IVIS 200 Imaging System (Revvity, Waltham, MA, USA).

5. Western Blot

Cells were lysed in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.05% SDS and 1% Igepal. Protein concentrations were determined using the Quick Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (50 μ g per lane) were separated by SDS-PAGE and subsequently transferred onto nitrocellulose membranes. Following transfer, EGFR, Nectin-4 and TROP-2 were detected using polyclonal rabbit anti-human EGFR IgG (#sc-03-G, Santa Cruz Biotechnology, Dallas, TX, USA), polyclonal rabbit anti-human Nectin-4 IgG (#17402S, Cell Signaling Technologies, Danvers, MA, USA), and monoclonal rabbit anti-human TROP-2 IgG (#90540, Cell Signaling Technologies, Danvers, MA, USA). Horse radish peroxidase (HRP)-conjugated polyclonal goat anti-rabbit IgG (#P0448, Dako Denmark A/S, Glostrup, Denmark) was used as secondary antibody. β -actin was detected as loading control using an HRP-conjugated mouse anti-human β -actin antibody (#HRP-66009, Proteintech Group Inc., Rosemont, IL, USA). Signals were visualized using an enhanced chemiluminescence (ECL) detection system and imaged with an INTAS Chemo Star Imager (INTAS Science Imaging Instruments, Göttingen, Germany).

6. Flow Cytometry

Cell binding of the antibodies and antibody dye conjugates to EGFR-, Nectin-4 and TROP-2 positive BC cell lines (RT112 and RT4) and to the antigen-negative control cell line CHO was analyzed by flow cytometry. For this, 2×10^5 cells/well were seeded and incubated with 10 μ g/ml of each antibody or antibody dye conjugate in a dilution buffer containing PBS with 3% fetal bovine serum and 0.1% sodium azide for 1 hour in the dark. A human IgG isotype control (Invitrogen, Karlsruhe, Germany) was used as negative control. After washing, cells were incubated with goat anti human IgG-PE secondary antibody (Southern Biotech, Birmingham, AL, USA) (1:500) and eBioscienceTM Fixable Viability Dye eFluorTM 450 (Invitrogen, Karlsruhe, Germany) (1:1000) for 30 minutes at 4°C in the dark. Following a final washing step, the cells were resuspended in the dilution buffer and analyzed using a FACSymphonyTM A1 Flow Cytometer (Becton Dickinson, Heidelberg, Germany). Mean fluorescence intensities (MFIs) were calculated using FlowJoTM software (Becton Dickinson).

7. Photoimmunotherapy

Target cells were seeded at a concentration of 1.25×10^5 cells/ml into 35 mm cell culture dishes (Thermo Fisher Scientific, Waltham, MA, USA) and cultivated for 24 h at 37 °C and 5% CO₂. On the following day, cells were treated for 24 h with either 10 μ g/ml of the antibodies Cmb^{T120C/D265C}, Enf^{T120C/D265C} or Sac^{T120C/D265C}, their corresponding conjugates, or an equimolar concentration of free dye WB692-CB2. Untreated cells served as controls. After treatment, cells were washed three times with PBS and phenol-red free medium was added before irradiation. Irradiation was performed using a light-emitting diode ($\lambda = 690$ nm; LED L690-66-60, Marubeni, Tokyo, Japan) at varying light doses ranging from 0 to 128 J/cm². For combinatorial PIT, RT112 and RT4 cells were treated with 10 μ g/ml of Cmb^{T120C/D265C}-WB692-CB2 or Enf^{T120C/D265C}-WB692-CB2 alone or in combination and irradiated with 64 J/cm² red light.

Twenty-four hours after PIT, cells were trypsinized, stained with Erythrosin B (Logos Biosystems, Gyeonggi-do, South Korea), and the numbers of living cells were determined using a Neubauer Counting Chamber. Number of living cells of the untreated and non-irradiated control

was defined as 100%, and numbers of living cells for the different treatment conditions were calculated relative to this control. Data represents mean values $\pm$ SD from three independent experiments. Statistical analysis were calculated using an unpaired Student's *t*-test with Welch's correction in GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA) with $p < 0.05$ considered statistically significant.

5. Conclusions

In summary, our study demonstrates that EGFR, Nectin-4, and TROP-2 are effective targets for PIT of NMIBC using cysteine-engineered antibody–dye conjugates. Among them, the TROP-2–directed conjugate Sac^{T120C/D265C}-WB692-CB2 showed the highest cytotoxic efficacy. These findings highlight PIT as a promising, targeted, and minimally invasive therapeutic approach for NMIBC that could complement or replace current intravesical treatments.

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Abbreviations

The following abbreviations are used in this manuscript:

ADC	antibody drug conjugate
BC	bladder cancer
BCG	bacillus calmette-guérin
Cmb	Cetuximab
DAMPs	damage-associated molecular patterns
EGFR	epidermal growth factor receptor
Enf	Enfortumab
EpCAM	epithelial cell adhesion molecule
EV	Enfortumab Vedotin
HER-2	epidermal growth factor receptor 2
ICI	immune checkpoint inhibitor
MIBC	muscle-invasive bladder cancer
NMIBC	non–muscle-invasive BC
PDT	photodynamic therapy
PIT	photoimmunotherapy
PS	photosensitizer
PSMA	prostate specific membrane antigen
ROS	reactive oxygen species

Sac Sacituzumab
TF tissue factor
TROP-2 trophoblast cell-Surface antigen 2

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