

Nanobody-photosensitizer conjugates for the treatment of epithelial ovarian cancer through targeted photodynamic therapy

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ABSTRACT

In this study, the concept of targeted photodynamic therapy, a method to induce cell death, was tested for epithelial ovarian cancer. Therefore, a recombinant single-domain antibody (7D12) was conjugated site-specifically to the photosensitizer IRDye700Dx by sortase-mediated transpeptidation (sortagging) and click chemistry. Cell binding and phototoxicity of these conjugates was evaluated in vitro in three different cell lines with different levels of epidermal growth factor receptor (EGFR)-expression.

It was demonstrated that targeting of IRDye700Dx with 7D12 lead to specific binding of these conjugates to high EGFR-expressing cells, allowing cell killing of only these cells after illumination of the photosensitizer.

Keywords

Epithelial ovarian cancer, photodynamic therapy, recombinant single-domain antibodies, cell killing

INTRODUCTION

Epithelial ovarian cancer is one of the four leading causes of cancer-related death in women. The disease is characterized by a general asymptomatic presentation, which leads to a late diagnosis. Due to this reason, most women show at least extensive abdominal spread when the disease is detected, leading to a poor prognosis. Patients with advanced stage ovarian cancer are routinely treated with surgery and adjuvant chemotherapy. [1, 2] Despite this aggressive standard treatment, the 5-year survival rate of these advanced stage patients is no more than 30 percent. [3] Therefore, more effective treatment modalities are absolutely needed. [1]

The concept of photodynamic therapy

Photodynamic therapy (PDT) is a method to induce cell death through administration and activation of

a so-called photosensitizer (PS) by application of light of a particular wavelength to the diseased area. Activation of the PS results in the formation of reactive oxygen species (ROS), leading to cell death. [4] PDT promises to become of great importance for cancer treatment in the future and is potentially a more effective treatment modality for epithelial ovarian cancer compared to the standard treatment. [5]

In this study, the concept of PDT was tested for epithelial ovarian cancer because patients show especially extensive abdominal spread and PDT may be applied locally in the peritoneum. It is of major clinical relevance that the tumors are accessible for the PS molecules as well as for light application. Both aspects are given by applying PDT locally in the peritoneum, making ovarian cancer suitable for treatment with PDT.

Improving tumor selectivity with targeting molecules

One challenge concerning PDT is a lack of tumor selectivity. The preferential uptake of the PS by malignant tissues is often not selective enough, leading to off-target toxicity as well. [6] To improve the tumor selectivity, special delivery systems and targeting molecules can be used for targeted delivery of the PS to the tumor.

One possible solution, which combines improved tumor selectivity with a more rapid clearance of unbound conjugates in comparison to monoclonal antibody (mAb)-PS conjugates, is the usage of recombinant single-domain antibodies (so called nanobodies (NBs)) for targeted delivery. [7] NBs consist of a monomeric antibody domain, which still has full antigen binding capacity. [4]

In ovarian cancer, there are several tumor-associated antigens known. One of them is the epidermal growth factor receptor (EGFR). [2]

The aim of this study was to develop PDT agents for future treatment of ovarian cancer by conjugating a NB targeting EGFR (7D12) site-specifically to the PS IRDye700Dx. Cell binding and phototoxicity of these conjugates was evaluated in vitro.

Based on the literature it can be hypothesized that targeting of the PS with a NB would lead to specific

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binding of the conjugates to EGFR-expressing cell lines allowing cell killing of only these cells after illumination of the PS.

METHODS

Cell lines

Three different cell lines with different levels of EGFR-expression were used in this project. The human epidermoid squamous carcinoma cell line A431 (high EGFR expression), the human ovarian carcinoma cell line SKOV-3 (moderate EGFR expression) and the human E98 glioma cell line were cultured in Dulbecco's Modification of Eagle Medium (DMEM) containing 4.5 g/L glucose and L-glutamine and supplemented with 40 µg/ml gentamicin and 10% Fetal calf serum (FCS) at 37°C and 5% CO₂.

Production and purification of NBs

The NB 7D12 was produced with a pelB leader sequence at the N-terminus and a C-terminal LPETG sequence and His-VSV tag by *Escherichia coli* ER2566 cells, transformed with pHENIX-7D12-LPETG-HIS-VSV plasmid. Standard protocols for extraction of periplasmic proteins were followed and purification of the his-tagged NBs was performed using NI-NTA sepharose beads and BIO-RAD columns, because only his-tagged proteins are able to bind to those beads. The protein concentrations were measured with Nanodrop proteinA280 and purity was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 15% gels. All gels were imaged with an Odyssey Infrared scanner. Purity was also analyzed by liquid chromatography-mass spectrometry (LC-MS; HPLC part: Shimadzu HPLC, MS part: Thermo Finnigan LCQ Fleet).

Synthesis of 7D12-IRDye700Dx conjugates

The 7D12-IRDye700Dx conjugates have been produced by sortase-mediated transpeptidation (sortagging) and click chemistry. This is a two-step reaction in which two already correctly folded proteins can be covalently linked resulting in an unnaturally linked fusion protein. First, the NB 7D12-LPETG-His-VSV was functionalized with the click chemistry tool H₂N-PEG₄-DBCO using WT sortase (Addgene, Plasmid #21931). Subsequently, the photosensitizer IRDye700Dx, available as NHS ester (hereafter named PS), was functionalized with another chemical reactive group, the H₂N-PEG₃-N₃.

These chemical reactive groups can then be used for copper free click chemistry. This refers to a reaction in which an azide (N₃) reacts with a strained cyclooctyne, like dibenzocyclooctyne (DBCO), thereby forming a covalent bond. Therefore, the PS-solution was incubated with the NBs at a 2-fold excess of PS-PEG₃-N₃ to VHH-LPET-PEG₄-DBCO ratio. All solutions were washed using Amicon® centrifugal filter devices

and efficiency of the click chemistry reactions were examined using SDS-page.

Cell binding experiments with the 7D12-PS conjugates

Fluorescent microscopy (EVOS) was used to examine whether the 7D12-PS constructs retained cell binding capacity. SKOV-3 and A431 cells as well as E98 cells, as negative controls, were detached with trypsin and seeded in 96-well plates (3000 cells/well; E98: 10 000 cells/well) overnight. After 1 h incubation with the 7D12-PS conjugates (4 µM) at 4°C, the cells were washed three times with PBA to remove unbound conjugates. Binding efficiency was analyzed with an Odyssey Infrared scanner and fluorescent microscopy (EVOS® FL Cell Imaging System) at 700 nm.

Cell viability assessment after PS treatment and LED illumination in vitro

The activity of all successfully synthesized conjugates was tested in vitro through light application- and light induced cell killing experiments. SKOV-3 and A431 cells as well as E98 cells, as negative controls, were detached with trypsin and seeded in 96-well plates (8000-20 000 cells/well). For each experiment, a duplicate plate was prepared as control without illumination. The cells were incubated with the 7D12-PS conjugates in various concentrations (100, 50, 10, 5 and 1 ng/µl) for 2 h at 37°C. After incubation, the media were changed to remove the unbound particles. Then, cells were exposed to LED-light (7.66 mW/cm² for 15 min, reaching a total light dose of 6,9 J/cm²). After illumination the cells were cultured further and the next day, cell killing was examined using MTT assays. Cells were incubated with 10 µl (added to cells in 100 µl media: 0.1%) of MTS-reagent (Promega, cat.no. G3580) in the incubator at 37°C and protected from light. 1h, 2h, 4h or 6h after adding the MTS-reagent, the ELISA reader at 482nm was used for measurements and the relative viability in percentage (%), related to cells without treatment, was calculated. In each experiment four wells were treated with the same concentration, making it possible to calculate an average and the standard deviation for each concentration. The results are expressed in different graphs using Excel and the GraphPadPrism 5.03 software for Windows.

RESULTS

Production and purification of NBs

After purification, only one remaining band can be observed in lane 5 on SDS page (Figure 1), displaying the purity of the obtained product. The results of LC-MS prove that the mass of this protein product correspond with the mass of 7D12-LPETG-His-VSV. Therefore, it can be concluded that production as well as purification have been successful.

Synthesis of 7D12-PS conjugates

After conjugating a click chemistry tool to the PS, those PS molecules were mixed with the functionalized NBs. Starting materials as well as the click reaction mixtures were analyzed by SDS-PAGE. The clear shift of the molecular weight after the click reaction indicates an increase in mass of the NB 7D12 and thus a successful conjugation of the PS to the NB (Figure 2). Only traces of free PS can be found in the samples after the second round of purification.

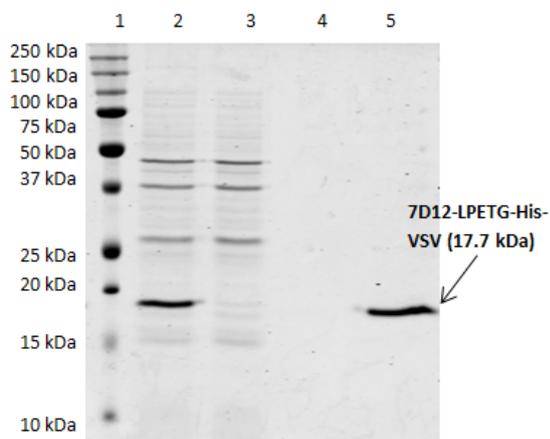


Figure 1: SDS-PAGE after purification of 7D12-LPETG-His-VSV (lane 5). The molecular weight marker, the non-purified product, the non-bound fraction and the pre-elution during nickel-bead purification are represented respectively in lane 1, 2, 3 and 4.

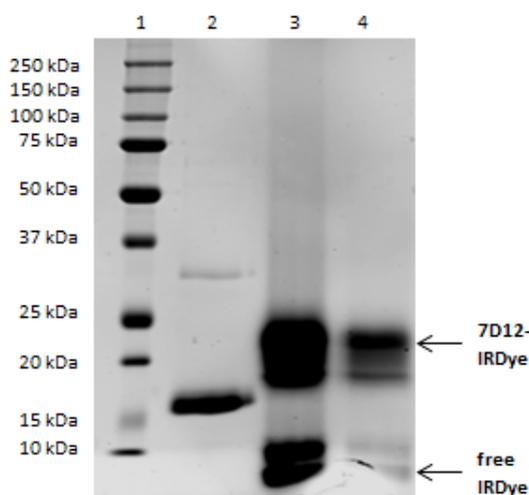


Figure 2: SDS-PAGE after click reaction between IRDye-PEG₃-N₃ and 7D12-LPET-PEG₄-DBCO (lane 4). Lane 1 represents the molecular weight marker and in lane 2 7D12-LPET-PEG₄-DBCO is shown. Lane 3 shows the reaction mixtures after the first round of purification. Lane 4 shows the product after the second round of purification.

Cell binding experiments with the 7D12-PS conjugates

Fluorescent microscopy (EVOS) was used to examine whether the 7D12-PS constructs have retained binding capacity. The compounds were incubated with SKOV-3 cells, with A431 cells as

positive control and with E98 cells as negative controls. Only after incubating the 7D12-PS conjugates with the A431 cells, a clear fluorescent signal could be detected with the EVOS.

Cell viability assessment after PS treatment and LED illumination (performed in Kuopio)

The 7D12-PS conjugates clearly lead to strong PDT-induced toxicity after incubation and illumination of the EGFR-positive A431 cells (Figure 3). With the two highest concentrations all cells are dead after treatment. After incubation with the EGFR-negative E98 cells this cell killing effect cannot be observed (Figure 3), indicating successful targeting of the 7D12-PS conjugates.

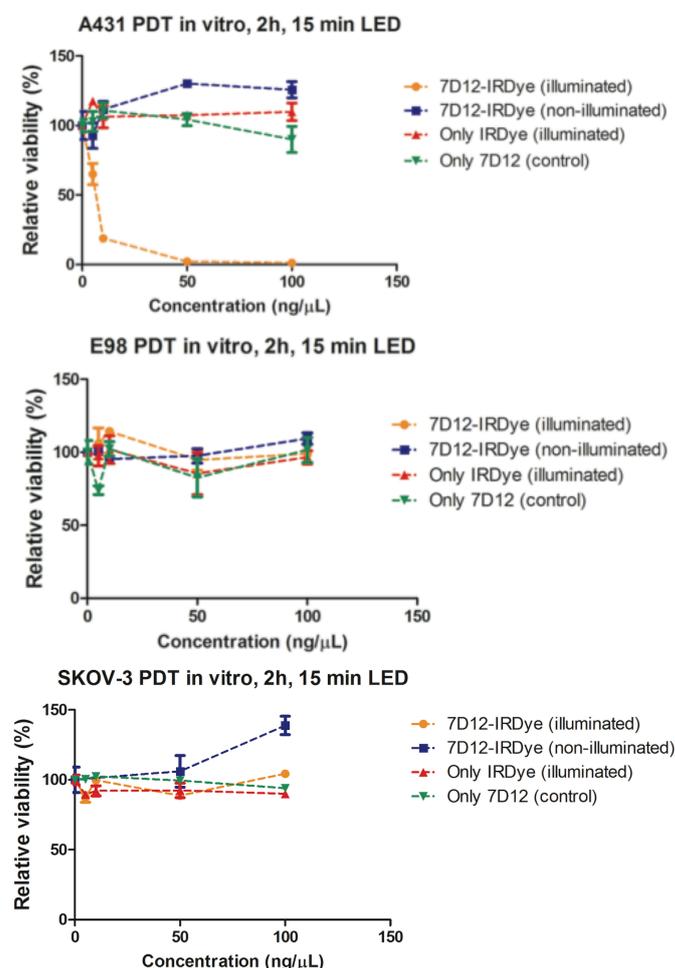


Figure 3: Results after PDT in vitro. A431 cells, E98 cells and SKOV-3 cells were incubated with a concentration range of 7D12-IRDye for 2 h and exposed to LED light for 15 min. The next day, the relative viability in percentage (%), shown on the y-axis, related to cells without treatment, was determined. On the x-axis the sample concentration (in ng/μl) is shown. Data are means and error bars are depicted.

In contrast to the A431 cells, with the SKOV-3 cells the cell killing effect is not that obvious (Figure 3). However, there is definitely a clear difference in relative cell viability between the illuminated experiments in comparison to the non-illuminated experiments. Without illumination there are more cells alive than after illuminating the

7D12-PS conjugates, indicating that there is an effect as well, only to a less extent.

Importantly, in all cell lines no dark toxicity was detected. That means that no cell killing occurred at the control plates without illumination. No cell killing was detected with free IRDye as well.

DISCUSSION

The concept of targeted photodynamic therapy (PDT) is a rapidly increasing research field, which promises to become of great importance for cancer treatment in the future. [5] Different approaches aimed to improve the tumor selectivity of the PSs by conjugating them to mAbs, but those targeting systems still have major drawbacks because of their long half-lives. [8] In this study, much smaller nanobodies have been used as targeting molecules, combining improved tumor selectivity with a more rapid clearance of unbound conjugates compared to mAb-conjugates.

The PS IRDye700Dx shows that it is a potent PDT agent because it can result in strong light induced cell-killing effects and importantly shows no dark toxicity.

The results show also that the concept of targeted PDT, tested in this project, works very well and the clear specificity of this PDT approach is underlined. Only very high EGFR-expressing cells are targeted by the 7D12-IRDye conjugates.

On the SKOV-3 cells, the cell killing effect is not that obvious but there is definitely a decrease in relative cell viability (Figure 3). These PDT results show a clear correlation with the results from the EVOS fluorescent microscopy. Already with the EVOS the binding to the EGFR-expressing SKOV-3 cells cannot be detected anymore. Only after treating A431 cells with the 7D12-PS conjugates, a clear fluorescent signal could be detected.

Most likely the amount of binding to the SKOV-3 cells seems to be too low, resulting in a detection problem with the EVOS. In addition, the amount of bound PS conjugates does not reach the threshold, which is needed for efficient working of PDT as well. In contrast, fluorescent microscopy shows a very high amount of 7D12-binding on the A431 cells, making it possible to reach the threshold amount of PS molecules which are needed for a strong light induced cell killing effect. These perceptions correlate very well with the characteristics of the cell lines. A431 cells are indeed characterized by an enormous EGFR over-expression.

CONCLUSION

All in all, it was demonstrated that targeting of the PS IRDye700Dx with 7D12 lead to specific binding of these conjugates to high EGFR-expressing cells allowing cell killing of only these cells after illumination of the PS.

ROLE OF THE STUDENT

For this research, I have been working as a bachelor student and participant of the Honours Programme (Radboud University Nijmegen) under the supervision of William Leenders. I performed all the experiments in collaboration with the rest of his team. This paper for the SRC application is a shorter version of my bachelor thesis and was fully written by myself.

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