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Nanobody–photosensitizer conjugates for targeted photodynamic therapy

Raimond Heukers, MSc, Paul M.P. van Bergen en Henegouwen, PhD, Sabrina Oliveira, PhD*
Molecular Oncology, Division of Cell Biology, Department of Biology, Faculty of Science, Utrecht University, Utrecht, the Netherlands
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Abstract

Photodynamic therapy (PDT) induces cell death through light activation of a photosensitizer (PS). Targeted delivery of PS via monoclonal antibodies has improved tumor selectivity. However, these conjugates have long half-lives, leading to relatively long photosensitivity in patients. In an attempt to target PS specifically to tumors and to accelerate PS clearance, we have developed new conjugates consisting of nanobodies (NB) targeting the epidermal growth factor receptor (EGFR) and a traceable PS (IRDye700DX). These fluorescent conjugates allow the distinction of cell lines with different expression levels of EGFR. Results show that these conjugates specifically induce cell death of EGFR overexpressing cells in low nanomolar concentrations, while PS alone or the NB–PS conjugates in the absence of light induce no toxicity. Delivery of PS using internalizing biparatopic NB–PS conjugates results in even more pronounced phototoxicities. Altogether, EGFR-targeted NB–PS conjugates are specific and potent, enabling the combination of molecular imaging with cancer therapy.

Key words: Photodynamic therapy; Targeted photosensitizer; Nanobody; VHH; Nanomedicine; Molecular imaging; EGFR

Background

Photodynamic therapy (PDT) makes use of three essential elements to induce cell death: a photosensitizer (PS), light of a particular wavelength, and oxygen. Since the first evidences of PDT-induced cell toxicity in the early 1900s,[1–3] many reports have been published on the usage of PDT to treat cancers of the bladder, skin, head and neck and of the ovaries, among others.[4–7] In general, the PS is administrated intravenously and, after a period of time, light of a particular wavelength is applied to the diseased area. The activated PS leads to type II photo-oxidative reactions, in which it reacts directly with oxygen to form the very toxic singlet oxygen ($^1O_2$) that damages lipids, proteins and/or nucleic acids[8]. Type I reactions can also occur, in which reactive oxygen species are formed via intermediate reaction of PS with substrates other than oxygen. As these transient oxygen species are short-lived molecules and have very short diffusion distances,[9] their toxicity is confined to the PS's localization upon light application. Subsequently, cells die through necrosis and/or apoptosis and tumor destruction occurs through micro-vasculature damage and involvement of both immune and inflammatory systems.[4] PSs clinically available are mainly derivatives of porphyrin (e.g. Photofrin®), chlorine (e.g. Foscan®), and phthalocyanine (e.g. Photosense®).[6]

In general, the relatively high degree of hydrophobicity and lack of specificity of the PS result in illumination times 2 to 4 days after PS administration, in some off-target toxicity, and in a rather long period of patients' photosensitivity after PDT treatment.[6,7] Therefore, efforts have been made to render PS more hydrophilic and to target these molecules more selectively to tumors, through chemical modifications, delivery systems, and/or targeting molecules.[10–14] In particular, photoimmunotherapy (PIT) refers to the use of monoclonal antibodies (mAbs) for targeting of PS in PDT.[12] Although promising results have been reported with mAb–PS conjugates,[15–18] these conjugates have long half-lives. Thus, further improvements would be valuable with respect of time needed for their tumor accumulation and the clearance of unbound conjugates. This has stimulated numerous studies on the usage of antibody fragments to target PS (e.g. Refs. [19–23]). With the same aim, we have developed conjugates that combine for the first time nanobodies (NBs) with a PS.

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*Corresponding author at: Molecular Oncology, Division of Cell Biology Department of Biology, Faculty of Science, Utrecht University Padualaan 8, 3584 CH Utrecht, the Netherlands.
E-mail address: s.oliveira@uu.nl (S. Oliveira).

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NBs are the variable domain of a particular sort of antibodies, i.e. the heavy chain-only antibodies that were first discovered in dromedaries in 1993.[24] Nanobodies can be considered as the smallest naturally occurring binding domain, that is approximately 10 times smaller than conventional antibodies (NBs: 15 kDa, 2.5 nm × 4 nm[25]; mAbs: 150 kDa, 14.2 nm × 8.5 nm × 3.8 nm[26]). Despite their size, NBs can bind very specifically and tightly to their antigens (low nanomolar affinities), such as the epidermal growth factor receptor (EGFR), which is overexpressed in many types of human cancers.[27] Recently, we have demonstrated the advantages of NBs for optical molecular imaging of EGFR-positive tumors.[28] EGFR-targeted NBs showed a faster accumulation at the tumor, a more homogeneous distribution within the tumor, and a more rapid clearance of unbound molecules, compared to an anti-EGFR monoclonal antibody. In an attempt to translate these properties to the PDT context, we have conjugated the same NB targeting EGFR (7D12) to a PS. Furthermore, similarly to what was shown with internalizing mAbs,[29–31] we aimed to improve the potency of the PDT even further by stimulating intracellular delivery of the PS. For that, we used a biparatopic NB (7D12-9G8) that is known to be internalized via clustering-induced endocytosis of EGFR.[32]

To further contribute to a more effective PDT, the PS used in this study is traceable through optical imaging, which enables light application at the most appropriate time and location. The idea of visualizing tumors through imaging of a PS dates back to the 1920s,[33] but the exploration of this feature is still in its infancy,[34] mainly due to the poor absorption of most PS in the near-infrared range, which is the most effective range of wavelength to penetrate through human tissues. The PS used in this study is the recently described, near-infrared fluorescent PS, IRDye700DX.[35] This silicon–phthalocyanine derivative is relatively hydrophilic, has the typical strong absorption band of phthalocyanines in the red region of the spectrum and the flexibility to be conjugated to proteins.[36] It has previously been conjugated to an EGFR-targeted mAb and was shown to be phototoxic when bound to the cell membrane or after internalization. Furthermore, tumor-specific PDT was shown, where shrinkage of tumors was only observed in those overexpressing EGFR.

In this study, we have conjugated monovalent and biparatopic NBs targeting EGFR to the traceable PS IRDye700DX. These conjugates are characterized and their phototoxicity is evaluated in vitro. These NB–PS conjugates could have a significant impact on current PDT protocols, combining molecular imaging with therapy.

Methods

Nanobodies and PS conjugation

Nanobodies (NBs) 7D12, R2, and 7D12-9G8 were produced as described in the Supplementary Materials. The photosensitizer IRDye700DX (here named PS) was purchased from LI-COR (LI-COR Biosciences, Lincoln, Nebraska) as an *N*-hydroxysuccinimidine (NHS) ester. Conjugation of the PS to the NBs, purification and characterization of the NB–PS conjugates were performed as described in the Supplementary Materials.

Cell lines and culture conditions

The mouse fibroblast cell line NIH 3T3 2.2 (abbreviated 3T3 2.2) was described in Ref. [37]; the human head and neck squamous cell carcinoma cell line UM-SCC-14C (abbreviated 14C) was kindly provided by Prof. Dr. G.A.M.S. van Dongen, (VUMC, Amsterdam, the Netherlands); the human epithelial carcinoma cell line A431 (CRL-1555) and the human cervical carcinoma cell line HeLa (CCL-2) were both obtained from ATCC (LGC Standards, Wesel, Germany). All cell lines were cultured as described in the Supplementary Materials.

Cell binding assay

Binding assays were performed on all cell lines, as described in detail in the Supplementary Materials. For evaluation of the association kinetics, 14C cells were incubated with 25 nm of NB–PS at 37 °C for up to 30 min. Thereafter, cells were washed twice and the fluorescence intensity (F.I.) of bound conjugates was detected with the Odyssey Infrared scanner, using the 700-nm channel.

In vitro PDT

One day after seeding 8000 cells per well of 96-wells plates (Greiner Bio-One, Alphen a/d Rijn, the Netherlands), cells are washed once with PDT medium (DMEM without phenol red supplemented with 8% FCS (vol/vol), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine). Then, a dilution range of NB–PS conjugates (or the 1:1 mixture of 7D12-9G8 with 7D12-9G8-PS) was added to the cells and incubated for 30 min at 37 °C. After the incubation (also referred to as pulse), cells were washed twice with PDT medium. Immediately after, the F.I. of the conjugates bound to and/or internalized by the cells was detected with the Odyssey scanner and the cells were illuminated immediately after, unless otherwise mentioned. Plates were illuminated with ~4-mW/cm^2 fluence rate (measured with an Orion Laser power/energy monitor, Ophir Optronics LTD, Jerusalem, Israel), for a total light dose of 10 or 5 J/cm^2, using a device consisting of 96 LED lamps (670 ± 10 nm, 1 LED per well) described in Refs. [38,39] 12After illumination, cells were placed back into the incubator, unless mentioned otherwise. In all experiments, a number of wells were covered during illumination as internal negative control. Experiments were repeated at least twice.

Cell viability assays

After overnight incubation of the cells treated as described above, cells were incubated with the Alamar Blue reagent, according to the manufacturer's protocol (AbD Serotec, Oxford, United Kingdom) and as described in the Supplementary Materials. Results are expressed as cell viability in percentage (%), thus relatively to the untreated cells, and the half maximal inhibitory concentration (IC50) are determined with using the GraphPad Prism 5.02 software.