Development of Nanobody Targeted Liposomes Directed Against Tumors for Image-Guided Drug Delivery

Michiel Bolkestein1,2, Dubravka Drabek3, Timo L. ten Hagen2, Marion de Jong4, Frank G. Grosveld3, Gerben A. Koning1,2

1Innovative Targeting Group, 2Laboratory Experimental Surgical Oncology, Department of Surgery; 3Department of Cell Biology; 4Department of Nuclear Medicine and Radiology
Erasmus Medical Center, Rotterdam, 3015CN, The Netherlands
m.bolkestein@erasmusmc.nl

Abstract Summary
To promote tumor cell targeting and intracellular drug delivery liposomal formulations with novel nanobody targeting modalities (nanobody-liposomes) have been developed. In combination with optical and nuclear probes these nanobody-liposomes enable the monitoring of tumor accumulation and can specifically induce therapeutic efficacy.

Introduction
In response to high toxicity and lack of efficacy of anti-cancer drugs, liposomal formulations have been successfully developed. These nanocarriers however show limited tumor accumulation and fall short of cell-specific intracellular delivery of their contents. We aim to optimize liposomal chemotherapy by increasing nanoparticle tumor accumulation with the use of cell-specific targeting modalities.

Figure 1: Enhanced tumor cell specific active targeting by nanobody-liposomes. (A) extravasation due to enhanced permeability in tumor vasculature, (B) tumor cell specific binding of targeted liposomes, (C) internalization of nanobody-liposomes, (D) intracellular drug release.

Tumor cell-specific targeting enables localized nanocarrier retention at and internalization by tumor cells (Figure 1). In this project we designed liposomes equipped with single-domain antibodies (nanobodies), i.e. antibodies containing only the variable domain of the antibody's heavy chain. These nanobodies will be targeted against various tumor specific antigens, such as EGFR and MUC1. EGFR is overexpressed in many tumor types, whereas MUC1 is more prominent in pancreatic cancer. Since these targets are tumor cell specific, it enables cell specific drug delivery. In addition, to be able to follow the fate of targeted liposomes in vivo and to ascertain tumor accumulation, liposomes were labeled with near infrared fluorophores or radionuclides.

Experimental Methods
A transgenic mouse has been developed, which enables the production of nanobodies of human origin (1). After immunization, antibodies were isolated and expressed, and nanobodies against MUC1 and EGFR (2) were generated. Nanobodies against MUC1 are still in a developmental phase and being tested for affinity against the antigen alone and against MUC1 expressing tumor cells. EGFR-nanobodies were validated for affinity against EGFR overexpressing cell lines, such as A431 (squamous cell carcinoma) and others, by flow cytometry and were used for site-directed coupling to liposomes. These nanobodies were produced as a complex with a His-tagged SUMO3 protein, which generates native nanobodies after cleavage with a SUMO-specific protease. The addition of a Cys-tag to the nanobody enables coupling to maleimide-lipids and presents the antibody's binding sites outwards, ensuring use of the full binding potential of the nanobodies. Liposomes were made according to the lipid film hydration and extrusion method, after which IRDye-800CW or 111Indium were attached to the bilayer and nanobodies were coupled to PEG chains by post attachment, using thiol-maleimide reactions. It is known that the permeability of tumors is
variable and that certain tumors show limited to no enhanced permeability and retention (3). Since this is vital to achieve tumor cell targeting the used tumor cell lines will be tested in vivo for liposome accumulation. To this end, pilots have been performed to ascertain tumor accumulation and biodistribution of $^{111}$Indium or IRDye-800CW labeled non-targeted liposomes of circa 90 nm in size, which were followed over several days with SPECT/CT or IVIS Spectrum, respectively.

RESULTS AND DISCUSSION

MUC1 antibodies were isolated and produced, and have been tested in HCAb (heavy-chain-only antibody) format displaying affinities in the nanomolar range. Currently these antibodies are being transferred into the nanobody format containing a Cys-tag for liposome conjugation. Nanobodies against EGFR have been produced in the SUMO3 expression system and tested positive (Figure 2) in vitro against a selection of EGFR positive cell lines amongst which are A431 (squamous cell carcinoma), and CFPAC, BxPc-3, and HPAF-II (adenocarcinomas). All apart from A431 also have MUC1 expression, which will be used to validate the MUC1-nanobodies.

Figure 2: Flow cytometry binding of Cys-tagged EGFR-specific nanobodies against A431. (A) 0 µg/mL EGFR-nanobodies, (B) 5 µg/mL EGFR-nanobodies

After validation of EGFR-nanobodies, post attachment to the distal end of PEG chains on preformed liposomes has been performed and proven successful. The EGFR nanobody-liposomes are tested in vitro on the same set of EGFR positive cell lines, before in vivo localization studies with optical or nuclear imaging will be performed. Meanwhile strategies for linking optical and nuclear probes to liposomes have been developed. Imaging of labeled non-targeted liposomes demonstrated different levels of uptake between different tumor types, including some which show no uptake at all. In the latter situation a mild hyperthermia tumor treatment could induce tumor vascular hyperpermeability and subsequent nanoparticle accumulation (4).

CONCLUSION

Functional novel antibodies against MUC1 have been produced and are being developed into nanobody format suitable for liposome targeting. Liposomes targeted to EGFR have been developed to enable internalization of liposomes and are currently being optimized for in vivo applications. Both targeted formulations are expected to increase specific accumulation of drugs into tumor cells and thus efficacy of drug-loaded liposomes. In addition, the imaging pilots with non-targeted liposomes show that tumor permeability is variable and affects tumor accumulation of non-targeted, as well as targeted liposomes. Mild hyperthermia can be used to induce tumor vascular hyperpermeability, also in tumors with intrinsically low permeability to liposomes. Thus, although targeted liposomal chemotherapy may aid specific intracellular drug delivery, hyperthermia may be needed to permeate tumor vasculature to allow access of these targeted liposomes and to target receptors on tumor cells, after which intracellular drug delivery can increase anti-tumor efficacy.

REFERENCES


ACKNOWLEDGMENTS

Technical support was provided by the Applied Molecular Imaging Facility (Erasmus Medical Center, Rotterdam).