Chapter 6

Summary, discussion & future perspectives

In this thesis generic strategies for mono- and dual labeling are described, enabling the efficient development and quantitative characterization of mAbs and mAb conjugates *in vivo* by PET or optical imaging. These labeling strategies are particularly attractive in the development and the *in vivo* characterization of highly potent therapeutic mAbs to confirm their potential for selective and specific targeting of tumors and individual tumor cells. The power of dual-radiolabeling was illustrated in the development of ADCs containing the highly toxic tubulysin moiety, which had never been exploited in ADC approaches before. For such ADCs stability in blood is a prerequisite, because sequestration of the free drug in normal organs might cause unacceptable toxicity. Moreover, it is important that the tumor targeting capacity of the mAb remains preserved when carrying the toxic compound, while uptake in normal organs should be minimized. This crucial information can be obtained from preclinical studies with tumor-bearing nude mice, as shown in this thesis, but particularly also in future clinical trials when cross-reactivity with normal tissues is more an issue and tumor targeting might vary among patients. To allow clinical application, strategies for labeling of mAbs should be current good manufacturing practice (cGMP) compliant. In this thesis we describe the cGMP compliant coupling of ^{89}Zr and/or IRDye800CW to allow the tracking of mAbs and mAb conjugates by single- or dual mode optical and PET imaging.

In the introduction section of this thesis (**Chapter 1**) the history of antibody development and applications in oncology, including technical milestones, is reviewed. Monoclonal antibodies (mAbs) were originally developed as naked unconjugated therapeutics, mostly for selective inhibition of receptor tyrosine kinases or to modulate or block other critical membrane targets. The safety and therapeutic successes of this first generation mAbs has stimulated the development of next generation mAbs to allow for increased potency. Together with immune checkpoint mAbs, ADCs are currently attracting the most attention. In the last decade, the increase in ADC-development has led to 2 new FDA-approved ADCs and over 30 ADCs in clinical stage development[1].

ADCs consist of a highly toxic drug, a linker and a mAb. All three components of the ADC have their own crucial and ideal properties contributing to therapeutic potential and clinical impact. The mAb should be highly specific for the tumor antigen, which preferably should be overexpressed on tumor cells only and should internalize after binding of the mAb. The linker should be stable in the circulation to avoid sequestration of the drug in normal organs, and only inside the tumor cell the drug should be released. And finally, the highly toxic drug has to be conjugated and released from the mAb without losing its toxic potential. Despite the large number of clinically tested ADCs, the toxic compounds used in these conjugates

are limited to five different chemical classes of toxic molecules: maytansinoids, auristatin, calicheamicin, duocarmicin, and daunorubicin. Three-quarters of ADCs utilize either maytansinoid or auristatin payloads[2]. A reasonable explanation for the limited number of toxic compounds in clinical studies is the difficulty in finding a toxic compound that exhibits toxicity at nanomolar concentration, and that can be conjugated to a mAb in an aqueous solution. Besides this, the possibility of large scale chemical synthesis of these natural occurring highly potent compounds should be secured to allow fluency in clinical development, while a new toxic compound requires full characterization in preclinical studies before allowing its testing in the clinical phase. Development of ADCs with new toxic compounds is therefore challenging, but can provide promising new therapeutics.

Tubulysin is a highly toxic tubulin-targeting agent with a narrow therapeutic window. In **Chapter 2**, derivatives of tubulysin are used in the development of novel ADCs. The natural occurring tubulysin has recently been chemically synthesized, which enabled development of analogues with varying toxic potential and chemical properties. Besides the development of procedures to couple tubulysins to mAbs, the second goal of this study was to obtain information on the *in vivo* behavior of the resulting complete ADC, of both the mAb as well as the toxic compound, since this data provides crucial information on the tumor targeting potential and possible side effects when used in treating patients. Therefore, the selected tubulysin derivatives used in this study, TUB-OH (IC_{50} > 100 nmol/L) and TUB-OMOM (IC_{50}, 0.4-6 nmol/L), both contained a phenol group to allow radiolabeling with radioactive iodine through an electrophilic substitution reaction on the phenol moiety. The selected mAb in this study, trastuzumab, was labeled with ^{89}Zr, enabling characterization of the complete ADC. Procedures were developed to radiolabel the tubulysin derivatives with ^{131}I, and to convert them to active NHS esters for successful coupling to unlabeled or ^{89}Zr-labeled trastuzumab through direct conjugation to the lysine-NH_2 group. A linker was not used in this initial approach of ADC development, since the currently available linkers are theoretically all less stable than the direct conjugation to the lysines of the mAb. After purification of the compounds, radiochemical purity of the radiolabeled ADCs was between 96% and 98%, caused by co-elution of non-conjugated tubulysin with the ADC. The lipophilic nature of the tubulysins used in this study is most probably causing trapping of the free drug in the tertiary structure of the mAb molecule. Biodistribution studies with the dual-labeled conjugates provided valuable information on the stability of the ADC and on the tumor targeting potential. Blood kinetics of ^{131}I-TUB-OMOM-^{89}Zr-trastuzumab on the one hand assessed by measurement of the ^{131}I-signal and on the other hand assessed by measurement of the ^{89}Zr-signal did not show significant differences, proving the stability of the ADC *in vivo*.

Moreover, comparing the ^{89}Zr-signal of the dual-labeled ADC and ^{89}Zr-trastuzumab showed no significant differences in blood- and tumor-uptake. This indicates that coupling of TUB-OMOM to trastuzumab does not alter the pharmacokinetics and tumor-targeting properties of trastuzumab, and, consequently, that TUB-OMOM can be inertly coupled to trastuzumab. These biodistribution studies also revealed elevated uptake of ^{131}I in comparison with ^{89}Zr in colon and ileum content. This might be due to free drug present in the sample for injection, since administration of free ^{131}I-TUB-OMOM to tumor-free mice resulted in predominant accumulation in the colon and ileum content.

To test the antitumor effect of the ADC, efficacy studies using single-dose TUB-OMOM-trastuzumab were performed in mice bearing trastuzumab-sensitive (N87) or trastuzumab-resistant (JIMT) xenografts. Dose-dependent antitumor effects, including complete tumor eradications were observed in trastuzumab-sensitive tumors *in vivo*. This means that it is not necessary to apply cleavable linkers to have a therapeutic effect, an aspect that may simplify clinical application. TUB-OMOM-trastuzumab (60 mg/kg) displayed efficacy similar to ado-trastuzumab emtansine (15 mg/kg), yet was more effective than trastuzumab. Neither TUB-OMOM-trastuzumab nor ado-trastuzumab emtansine caused antitumor effects in mice bearing trastuzumab-resistant JIMT xenografts, despite the fact that TUB-OMOM-trastuzumab is targeting JIMT and N87 tumors equally well, as shown by the biodistribution results.

Labeling of both parts of the ADC provided knowledge on the efficiency and inertness of conjugation of the tubulysin to the mAb, the stability of the ADC *in vitro* and *in vivo*, the normal tissue and tumor uptake *in vivo*, and the excretion route of the tubulysin. Without the use of radioactivity, mass spectrometry would have been necessary to determine ADC composition and integrity, which is challenging in tissue samples, while no accurate quantitative information can be obtained about tissue uptake levels.

Despite the promising therapeutic results obtained with the TUB-OMOM-trastuzumab ADC, there is still room for further improvement. Firstly, SDS-PAGE analysis of the radiolabeled product revealed a small percentage of free tubulysin in the purified conjugate, which is not acceptable for clinical application. The most ideal solution to this problem would be to find a proper additive for removal of the weakly bound free tubulysin drug out of the tertiary structure of the mAb before purification. In that case our developed coupling procedure, giving a robust peptide bond that is stable in vivo, might well be the favorite for clinical application. If not, a possibility to solve this problem could be the use of the recently described innovative linker technology for coupling of supertoxic drugs to mAbs via platinum(II) as bifunctional linker (called "Lx" linker technology)[3]. In this ap-

proach, the Lx-drug used for conjugation is better water-soluble than the drug itself, making purification of the mAb-Lx-drug complex easier. This approach seems also applicable for coupling of tubulysins to mAbs. Secondly, the TUB-OMOM-trastuzumab ADC used in this study was not more effective than the reference ADC ado-trastuzumab emtansine in N87 xenograft bearing mice, while both were ineffective in JIMT xenograft bearing mice. But nowadays, tubulysin derivatives 10-100 times more potent than TUB-OMOM are available, so further improvement of ADC efficacy can be envisioned, possibly facilitated by the introduction of advanced linker systems. Obviously, during these optimization steps, dual-radiolabeling can be of paramount value.

Although the dual-labeling approach can provide particularly useful information in preclinical studies of new ADCs, we foresee that also in clinical development the labeling of the mAb-vehicle will still be beneficial: immuno-PET can reveal the ideal mAb dosing for optimal tumor targeting (e.g., saturation of receptors), the uptake in critical normal tissues to anticipate toxicity, and the inter-patient variation in pharmacokinetics and tumor targeting.

Imaging is not only important to confirm selective tumor targeting of mAbs and mAb conjugates at the whole body level, for some mAbs it might also be essential to know about their extravasation and tumor penetration and to identify cell subsets that become targeted. Assessment of tumor penetration might be relevant to confirm homogeneous distribution of the mAb throughout the tumor, which might be a prerequisite for mAb efficacy. Such analyses became technically more challenging by the introduction of human mAbs. Knowledge about targeting of cell subsets is valuable for mAbs that demonstrate cell type specificity, e.g., mAbs directed against tumor vasculature, immune checkpoint antigens or tumor stem cells. The combination of PET with near-infrared (NIR) can transform mAb imaging into true “whole body immunohistochemistry”.

NIR probes are promising candidates for imaging and they can have a complementary clinical potential to immuno-PET. First of all, they allow high-resolution, real-time, dynamic imaging of superficial tissue layers at the cellular level. Targeting at the cellular level can also be confirmed *ex vivo* by taking biopsies. What is more, they might be ideal for the detection and characterization of early-stage or residual disease, for example of cancer during surgery or in a screening setting. This would add a new dimension to the clinical application of mAbs.

The most promising NIR-dye is IRDye800CW, it can be functionalized with either an NHS or a maleimide group, enabling its coupling to a mAb. Encouraging preclinical imaging results with IRDye800CW-mAb-conjugates have been reported[4-8]. An important aspect of a NIR-dye, as with ADCs, is that it is a lipophilic molecule

that is conjugated to a hydrophilic mAb. Therefore, complete characterization of the IRDye800CW-mAb should be performed before clinical studies can start. It should be established that the binding characteristics, pharmacokinetics, and dynamics of the mAb do not become impaired upon coupling of the NIR-dye to the mAb.

In **Chapter 3**, we characterize the NIR-dye IRDye800CW coupled to two FDA approved mAbs: bevacizumab and cetuximab. To obtain quantitative reference data of these conjugates, ^{89}Zr-labeled mAbs were used, since stability, binding characteristics, and *in vivo* biodistribution of PET-tracers can easily and accurately be analyzed in a quantitative way based upon radioactivity readout. The number of chelator groups required for the ^{89}Zr-labeling was kept to a minimum of on average 0.5 groups per mAb molecule, to minimize the total number of coupled groups. Up to five IRDye800CW groups were coupled to the lysine residues of the ^{89}Zr-mAbs, followed by size exclusion chromatography giving conjugates that were more than 99% pure for ^{89}Zr as well as for IRDye800CW, while the integrity of the mAbs, as assessed by HPLC analysis, remained fully preserved. The immunoreactivity and stability of the ^{89}Zr-mAb-IRDye800CW conjugates remained preserved upon storage in 0.9% NaCl at 4°C for several days and storage in PBS and human serum at 37°C did not affect the stability for at least 96 h. Biodistribution studies, however, showed a faster blood clearance and increased liver uptake when more than on average 1 eq of IRDye800CW was coupled to both ^{89}Zr-cetuximab and ^{89}Zr-bevacizumab. This data indicates that to ensure unimpaired NIR-mAb conjugates for clinical studies not more than on average 1 eq of IRDye800CW should be coupled per mAb molecule, also when no dual labeling with ^{89}Zr is performed. We expect that this should not have great impact on the optical performances of the conjugate, since optical images of mAbs with on average 1 eq of IRDye800CW per mAb molecule showed clear tumor delineation. Earlier preclinical studies with dual-labeled mAb-conjugates containing IRDye800CW and a PET/SPECT-tracer, compared the radioactive signal with the fluorescent signal to draw conclusions on the stability of the conjugate. However, we show that even similar signals do not prove that labeling kept the mAb unaffected. After the publication of the findings described in this chapter, most subsequent research papers dealing with IRDye800CW-mAb conjugates followed our findings and coupled on average 1 eq of dye to the mAb[9-11]. Although inertness of the dual-labeled conjugate was tested for two FDA-approved mAbs in our study, extrapolation to other mAbs should be done with care. Every mAb has different properties and therefore stability and PK studies should be performed to confirm inertness of labeling with every new mAb.

At this moment it is not clear why coupling of just a few IRDye800CW molecules to lysine residues of a mAb has such a dramatic effect on pharmacokinetics. When

coupling $^{99m}Tc/^{99}Tc$-MAG3 or ^{186}Re-MAG3 chelate groups to lysine residues a similar effect on pharmacokinetics was seen, but only when 8 groups or more were coupled, while immunoreactivity only slightly decreased upon coupling of more than 12 groups[12]. Also different mAbs became differently affected, and a relationship with the baseline isoelectric point of the mAb seemed apparent. In contrast, alteration of pharmacokinetics at low molar ratios (~ 1) was also observed when the photosensitizer meta-tetrahydroxyphenylchlorin (mTHPC) was coupled via lysines to mAbs[13], but this photosensitizer is much more hydrophobic than IRDye800CW. These studies clearly show that depending on the nature of the mAb and the nature and number of chelate or dye molecules coupled, alteration in hydrophobicity, charge or conformation might be introduced, resulting in an altered behavior of the mAb conjugates *in vivo*. In this regard, it would be interesting to see how site-specific or site-restricted labeling would affect mAb characteristics upon coupling of IRDye800CW.

In **Chapter 4**, a protocol is provided for dual-labeling of mAbs with IRDye800CW and ^{89}Zr in a current cGMP-compliant way. IRDye800CW and ^{89}Zr are coupled inertly, without impairment of immunoreactivity and pharmacokinetics of the mAb. The protocol is divided in three parts, to allow for preparation of conjugates for both single-modal (PET or optical) and dual-modal (PET and optical) clinical imaging. Also, the protocol makes aware of critical steps and provides troubleshooting advice.

Optical imaging probes, like IRDye800CW-mAb conjugates are very suitable for qualitative imaging of subcutaneous tumors or intraoperative imaging. To acquire information on the capacity of the conjugate to target the tumor and the level of specific accumulation in the tumor, a method would be preferred that allows for accurate quantification of the conjugate in percentage of injected dose per gram of tissue (% ID/g), like with radiolabeled conjugates. Assessment of organ- and tumor-distribution of IRDye800CW-mAb conjugates has been done with intact organs *ex vivo* or by imaging of tissue sections, using the same systems as used for noninvasive imaging of mice. Quantitative biodistribution of fluorophores is very difficult, due to the possibility of quenching of the fluorescence when NIR-dye molecules are present at a high concentration and to the scattering and absorption of photons by tissue components. In **Chapter 5** a new method for *ex vivo* quantification of IRDye800CW fluorescence in tissues is described. To validate the method, it was compared with biodistribution studies using ^{89}Zr-labeled mAb or dual-labeled ^{89}Zr-mAb-IRDye800CW, for reasons mentioned earlier. Dual-labeled cetuximab, ^{89}Zr-cetuximab-IRDye800CW, was prepared, according to the protocol described in **Chapter 4**, with on average 0.5 chelator groups and 0.9 dye groups coupled per mAb molecule. After size exclusion chromatography, the dual-labeled cetuximab

was found to be more than 99% pure for ^{89}Zr as well as for IRDye800CW. For imaging purposes a mixture of ^{89}Zr-cetuximab + cetuximab-IRDye800CW was used, since PET imaging requires a higher radioactive dose than the biodistribution studies. PET and optical imaging were performed 24 h p.i. to confirm correspondence of the biodistribution by imaging. For the biodistribution study, organs and tumors were collected 24 h p.i., and each of these were cut in two halves. One half was used for the assessment of uptake by radioactivity measurement. The other half was homogenized, and the content of the fluorescent probe was determined by extrapolation from a calibration curve made with the injected probe. Similar results were obtained for uptake of the conjugate irrespective whether using the radioactive signal or the fluorescence signal, in tumors, liver, lung, stomach, skin, and intestines. Significant differences were obtained for the blood, spleen, sternum, and muscle, showing higher values for the ^{89}Zr-signal, while the kidney showed a higher value with NIR fluorescence quantification. Since control studies have shown the stability of the conjugate in serum, and as it is known that both ^{89}Zr and IRDye800CW residualize after receptor-mediated internalization of cetuximab, these variations are most likely due to liver catabolism. Overall, the results obtained by NIR fluorescence quantification are considered to be similar to the results obtained with the reference method employed for radiolabeled conjugates. This method for dye quantification will most probably be applicable to other NIR fluorescent conjugates, although residualization of the fluorophore should be confirmed as well as the stability of the conjugates in serum, since every mAb can behave differently.

The results obtained in **Chapter 2-5** show the power of mAb labeling and molecular imaging in the design, chemical development, preclinical evaluation and clinical application of mAbs and mAb conjugates. These are just examples, obtained with generic labeling and imaging tools that can be applied during each stage of the translational chain of mAb development. These tools might contribute to precision medicine: efficient development of the right drug for the right patient, avoiding lengthy and costly procedures of drug development with many patients involved.

REFERENCES

1. Mullard A. Maturing antibody-drug conjugate pipeline hits 30. Nat Rev Drug Discov 2013;12: 329-32.
2. Hamilton GS. Antibody-drug conjugates for cancer therapy: The technological and regulatory challenges of developing drug-biologic hybrids. Biologicals 2015.
3. Waalboer DC, Muns JA, Sijbrandi NJ, Schasfoort RB, Haselberg R, Somsen GW, et al. Platinum(II) as bifunctional linker in antibody-drug conjugate formation: coupling of a 4-nitrobenzo-2-oxa-1,3-diazole fluorophore to trastuzumab as a model. ChemMedChem 2015;10:797-803.
4. Yang Y, Zhang Y, Hong H, Liu G, Leigh BR, Cai W. In vivo near-infrared fluorescence imaging of CD105 expression during tumor angiogenesis. Eur J Nucl Med Mol Imaging 2011;38:2066-76.
5. Vermeulen JF, van Brussel AS, Adams A, Mali WP, van der Wall E, van Diest PJ, Derksen PW. Near-infrared fluorescence molecular imaging of ductal carcinoma in situ with CD44v6-specific antibodies in mice: a preclinical study. Mol Imaging Biol 2013;15:290-8.
6. Day KE, Sweeny L, Kulbersh B, Zinn KR, Rosenthal EL. Preclinical comparison of near-infrared-labeled cetuximab and panitumumab for optical imaging of head and neck squamous cell carcinoma. Mol Imaging Biol 2013;15:722-9.
7. Bhattacharyya S, Patel N, Wei L, Riffle LA, Kalen JD, Hill GC, et al. Synthesis and biological evaluation of panitumumab-IRDye800 conjugate as a fluorescence imaging probe for EGFR-expressing cancers. Medchemcomm 2014;5:1337-46.
8. Korb ML, Hartman YE, Kovar J, Zinn KR, Bland KI, Rosenthal EL. Use of monoclonal antibody-IRDye800CW bioconjugates in the resection of breast cancer. J Surg Res 2014;188:119-28.
9. Zhang Y, Hong H, Severin GW, Engle JW, Yang Y, Goel S, et al. ImmunoPET and near-infrared fluorescence imaging of CD105 expression using a monoclonal antibody dual-labeled with (89) Zr and IRDye 800CW. Am J Transl Res 2012;4:333-46.
10. Zhang Y, Hong H, Orbay H, Valdovinos HF, Nayak TR, Theuer CP, et al. PET imaging of CD105/ endoglin expression with a $^{61/64}$Cu-labeled Fab antibody fragment. Eur J Nucl Med Mol Imaging 2013;40:759-67.
11. Lütje S, Rijpkema M, Franssen GM, Fracasso G, Helfrich W, Eek A, et al. Dual-Modality Image-Guided Surgery of Prostate Cancer with a Radiolabeled Fluorescent Anti-PSMA Monoclonal Antibody. J Nucl Med 2014;55:995-1001.
12. van Gog FB, Visser GW, Klok R, van der Schors R, Snow GB, van Dongen GA. Monoclonal antibodies labeled with rhenium-186 using the MAG3 chelate: relationship between the number of chelated groups and biodistribution characteristics. J Nucl Med 1996;37:352-62.
13. Vrouenraets MB, Visser GW, Stewart FA, Stigter M, Oppelaar H, Postmus PE, et al. Development of meta-tetrahydroxyphenylchlorin-monoclonal antibody conjugates for photoimmunotherapy. Cancer Res 1999;59:1505-13.