Fluorescently labelled monoclonal antibodies for real-time molecular imaging

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CHAPTER 10

Summary, discussion and future perspectives

SUMMARY

Optical molecular imaging revolves around the *in vivo* assessment and measurement of biological processes at a molecular and cellular level, using fluorescent light signals emitted by a targeted tracer molecule. In this thesis, we aimed to provide an overview of the phases of development for fluorescent optical tracers, with a focus on the steps required to develop a laboratory-grade probe into an investigational medicinal product fit for use in phase 1 clinical trials.

Chapter 1 describes the general introduction to the topic of molecular imaging and specifically optical molecular imaging, and discusses the historical perspective of previously used imaging modalities using aspecific optical dyes as well as the transition to near-infrared dyes, targeted probes, and the emerging application of targeted optical tracers in humans, and the need for pharmaceutical development of these novel targeted tracers.

The first phase of development, consisting of laboratory-scale conjugation of the tracer and testing of the primary mode of action, is depicted in **Chapter 2** for the near-infrared targeted photodynamic therapy tracers cetuximab-IRDye700DX and trastuzumab-IRDye700DX. Both tracers were successfully conjugated to the dye, with a 4:1 dye to protein ratio. Experimental proof of the tracer concept was shown by binding the tracers to 4 different cell lines (2 from esophageal adenocarcinoma, 1 from non-small-cell lung cancer and 1 from human breast carcinoma) and exposing the tracer-bound cells to multiple intensities of NIR light. It was shown that both the presence of tracer antibody and the light dose are required to achieve high cytotoxicity. Compared to untreated cells, treatment with cetuximab-irdye700DX resulted in a cytotoxicity of 54%, significantly higher than for an equivalent dose of unconjugated dye (2%; P = 0.017), or when antibody binding sites were blocked by an excess dose of unconjugated cetuximab (6%; p = 0.017). Pretreatment of the cells with a tyrosine kinase inhibitor to induce expression of more EGFR and human epiderman growth factor receptor 2 (HER2) molecules resulted in up to 39% increase in cell death when performing NIR-tPDT with cetuximab-IRDye700DX and up to 24% increase when using trastuzumab-IRDye700DX. These initial in-vitro experiments show that EGFR- or HER2-targeted tracers for NIR-tPDT are a promising treatment strategy. In addition, these data provide the basic conjugation methods, chemical-pharmaceutical properties of the compound and a functional proof-of-principle. These data form the basis of optimization and characterization experiments in the second phase of development.

The second phase is aimed at more extensive characterization of the compound using specific protein-focussed methods to determine if the tracer still retains the core properties of the antibody it was based on, as well as on formulation and stability studies to optimize the shelf-life of the compound and on toxicity testing to assure its safety in preclinical and clinical trials. These development activities were shown for bevacizumab-800CW in **Chapter 3**. The initially developed conjugation process for this tracer resulted in labelling with a dye

to protein ratio of 4:1, and extended characterization showed that the original protein structure was intact after labelling, and the antibody could still bind to its target. A small animal toxicity study showed that the labelled drug had no significant toxic effects, when administered at 100x and 1000x the intended clinical microdose. A microdose for tracers was defined as 4.5 mg flat dose (based on the FDA guidance for industry on Exploratory IND studies, which defines 30 nmol as the microdose for proteins). The developed product was produced for each patient individually, but had only a short shelf-life, and showed formation of aggregates. Additional development was performed and the process was redesigned to use a lower dye to protein ratio of 2:1 in production, and formulations for the protein were tested to extend its shelf-life. After 1 month of preliminary stability, it was found that a buffered solution with sodium chloride or trehalose as isotonicity agent showed the best performance. A stability study was performed for both new formulations, showing that the labelled protein was intact, within 90-110% of its original concentration, and displayed low levels of impurities after 3 months storage in 50 mM sodium phosphate buffer at pH 7.0 at 2-8°C. This formulation was then selected for production of tracer for clinical trials. These results display additional development combined with clinical translation, as the stability study was designed to provide the data needed for the investigational medicinal product dossier (IMPD) and the clinical trial application, and the resulting formulation could be produced according to Good Manufacturing Process (GMP) guidelines.

Using bevacizumab-800CW produced under GMP, multiple phase I trials were performed, two of which are described in **Chapter 5 and 6**. First, **Chapter 5** describes a phase I dose-escalation study for bevacizumab-800CW, where 26 patients with invasive T1-T2 breast cancer who were scheduled for surgery were included. A single dose of bevacizumab-800CW (4.5 mg, 15 mg, 25 mg or 50 mg) was administered three days before breast-conserving surgical intervention. Tumour-specific targeting, and fluorescent visualization of this tracer was shown to be feasible through intra-operative imaging, *ex vivo* scanning of the whole resected lump, fluorescent imaging of serially sectioned ("breadloaf slices") tissue specimens of the resected lump, and in formalin-fixed paraffin embedded tissue sections on fluorescent flat-bed scan and on fluorescent microscopy. Of the 26 study participants, 8 were found to have a positive resection margin, that was not found through standard of care visual inspection and palpation. For 7 patients of this group of 8, the positive margin could have been determined through fluorescence imaging, representing an 88% increase in the intra-operative detection rate of tumour-involved margins. This chapter also describes an analytical framework to serve as a standardized evaluation method for clinical trial data from fluorescence guided surgery studies, consisting of (i) qualitative *in vivo* intraoperative macroscopic imaging to determine the potential clinical value of fluorescence-guided surgery, (ii) qualitative *ex vivo* imaging of the fresh whole surgical specimen, (iii) quantitative *ex vivo* imaging of the fresh tissue slices of

the specimen to determine the tracer distribution on a macroscopic level, (iv) quantitative multi-diameter single-fiber reflectance/single-fiber fluorescence (MDSFR/SFF) spectroscopy of the fresh tissue slices to determine the intrinsic fluorescence intensities, (v) quantitative fluorescence flatbed scanning and (vi) fluorescence microscopy of formalin-fixed paraffin-embedded (FFPE) blocks and 10-µm-thick sections to determine the tracer distribution on a microscopic level and fluorescence microscopy. The proposed analytical framework is robust and broadly applicable, and can support the development of a standard evaluation methodology for the use of fluorescent imaging trial data in clinical translation of fluorescent tracers.

The study described in **Chapter 6** shows bevacizumab-800CW used as a tracer for endoscopic detection of residual disease after neoadjuvant chemoradiotherapy (nCRT) of locally advanced rectal carcinoma in 25 patients. Management of locally advanced rectal carcinoma without surgical intervention is associated with high survival rates, low morbidity and improved outcome. Therefore, there can be value in determining the response to nCRT before performing surgery. Using the quantified intensity of the fluorescent signal measured *in vivo*, it was shown that fluorescent imaging would have changed the diagnosis of the patients in 4 out of 25 patients, potentially resulting in a different follow-up approach for the patient. Out of the 4, 3 patients showed clinical response on white-light endoscopy and MRI imaging, but also showed increased fluorescence. For 2 out of these 3, vital tumour was confirmed at histopathological evaluation, and the third showed rapid regrowth (after 2 months). The last of the 4 showed residual tumour on white-light endoscopy and MRI, but low fluorescence, and a pathological complete response was found on pathological examination. This shows encouraging results for the use of quantitative fluorescence endoscopy for tumour response evaluation following nCRT. The results from **Chapter 5 and 6** underline the feasibility of using optical imaging in both surgical and endoscopic settings, as well as at doses beyond microdose (4.5 mg). Results like these, along with the tested dosages and routes of application for the tracer, as well as the protocols for the preparation and administration of the tracer are collected into the product dossier which can be used to further optimize and fine-tune the product down the line. The resulting input-output dynamic forms a constant cycle of improvements/development and evaluation, in order to get an optimized product specifically fit for its clinical application, at which point development is finalized and the production process is frozen.

The clinical translation of tracers is a time- and resource-intensive process, due to the large array of extra tests needed and the documentation required for each step. Since specific guidance for the development of optical tracers was lacking, we set out to formulate a standardized approach for all required activities and associated reports to build an IMPD. A standardized methodology for performing these translation steps is provided in **Chapter 4** in the form of the optical tracer development and translation roadmap.

The roadmap describes the minimum requirements for the types and extent of data to be collected for several steps in the development of a product for clinical trials. The roadmap was applied to the development of cetuximab-800CW and trastuzumab-800CW. Both antibodies were conjugated successfully, and tested for formulation compatibility and preliminary stability. Cetuximab-800CW was tested in 7 formulations and trastuzumab-800CW was tested in 10 formulations. From the stability performance in this first study, 2 formulations were chosen for each antibody to be included in a full-scale stability study. Both cetuximab-800CW and trastuzumab-800CW were stable for 3 months when stored at 2-8°C, though both were conjugated and formulated in different ways. Cetuximab-800CW was conjugated at a dye to protein ratio of 2:1 and formulated in 50 mM phosphate buffer, pH 7.0. Trastuzumab-800CW was conjugated at a dye to protein ratio of 1:1 and formulated in 10mM phosphate, pH 7.0. By applying the roadmap, both antibodies had sufficient data available to support clinical phase 0/1 microdose trials. Results of the clinical trials with cetuximab-800CW can be found in the contributions by Voskuil et al.(1) and Vonk et al. (2) The roadmap was designed to be applied to other antibody-based tracers as well, and to be used as basic guidance for researchers during the development a product that is suitable for human use. As such it also fills a gap in available official guidelines on optical tracer production.

The final part of this thesis applied the standardized methodology used for bevacizumab-800CW and cetuximab-800CW to two new fields for optical imaging. First, the potential for dual-wavelength imaging during checkpoint inhibitor therapy was investigated. **Chapter 7** describes the initial development and characterization of a pair of tracers that can be used for simultaneous imaging of PD-1 and PD-L1, two proteins in the checkpoint inhibition axis. This chapter shows the successful development of durvalumab-680LT and nivolumab-800CW. By targeting both of these receptor proteins with different tracers, both the tumour cells that overexpress PD-L1 and the infiltrating immune cells that express PD-1 can be imaged, and areas where interaction between both types of cells takes place will show up as overlap in the fluorescent signals. Nivolumab and durvalumab both labelled to their fluorescent dyes with high efficiency of 76.68% - 88.42%, showed no aggregates and <3% unconjugated dye. The conjugation process was optimized with regard to time and the dye to protein ratio at conjugation. The final process used a molar ratio of 2 dyes per protein and an incubation period of 1 hour. A formulation study yielded multiple feasible buffer candidates for both tracers. Durvalumab-680LT was prioritized for further development, and a stability study up to 6 weeks after production was performed. A formulation of 50 mM sodium phosphate buffer with sodium chloride at pH 7.0, either with or without 0.02% polysorbate 80, was shown to result in a stable product. Durvalumab-680LT protein content was within 90 – 110% of the original value, impurities were below 3%, and target binding capacity was maintained. The data presented in this chapter form the

basis for an extended, full-scale formulation and stability study, according to the roadmap from chapter 4. This will allow durvalumab-680LT to be translated to a product for phase I studies.

The second new field we investigated was the use of optical imaging in inflammatory bowel disease (IBD). **Chapter 8** describes the conjugation of four biological drugs licenced for use in IBD (infliximab, vedolizumab, adalimumab, ustekinumab) to 3 different fluorescent dyes (IRDye 800CW, IRDye 680LT, ZW800-1), and evaluation of their potential as a tracer for IBD. We found that conjugates of infliximab did not retain their intact antibody structure after dye conjugation, regardless of which dye was used. Antibody conjugates with ZW800 were considerably less bright, showed reduced yield after purification and showed formation of protein fragments. Out of the 12 tested tracer candidates, only 5 were considered feasible for further development and translation: vedolizumab-800CW, vedolizumab-680LT, adalimumab-800CW, adalimumab-680LT and ustekinumab-800CW.

Of the 5 tracers considered feasible in **Chapter 8**, vedolizumab-800CW was the first one to be selected for further development. Vedolizumab-800CW was fully translated and produced under full GMP conditions (not described in this thesis) for human use in a clinical trial setting. **Chapter 9** describes the application of vedolizumab-800CW in a phase I clinical trial. Patients who had an established diagnosis of IBD but were naïve to vedolizumab, were included and given a tracer dose of 0.0 mg (control group), 4.5 mg, 15.0 mg or 15.0 mg preceded by a "blocking" dose of 75.0 mg unlabelled vedolizumab. We showed in a preliminary report of the first 28 patients that fluorescent signal from vedolizumab-800CW was visible in the mucosa of IBD patients during endoscopy, and could also be detected in biopsy material. The wide-field image *in vivo* gave a diffuse, overall low-intensity fluorescent signal but intra-individual differences could be observed between healthy and inflamed tissue during endoscopy. MDSFR/SFF spectroscopy was additionally used to quantitatively measure intrinsic fluorescence, and showed a clear increase in signal related to the degree of inflammation for all dose groups except the control. This first report paves the way for more in-depth investigation into the microscopic distribution of the tracer, as well as investigation of the cells affected by vedolizumab. The trial will additionally be continued to show the mucosal fluorescence in patients who have already received therapy with vedolizumab, and will include patients that were imaged when they were naïve to vedolizumab a second time to evaluate any changes to mucosal fluorescence, and to correlate imaging results to clinical follow-up. These data will provide new insight into the mechanism behind vedolizumab's pharmacodynamics, and may be used to support the management of vedolizumab in clinical practice in the future.

DISCUSSION

Fluorescent molecular imaging has begun to take flight in the past decade. Since the first trials in ovarium carcinoma (3), multiple studies have been performed that show potential benefit for other fields (1,4–10). Most of these are within the oncology disciplines, but a shift towards inflammatory disorders, like inflammatory bowel disease and arthritis, has recently also been shown. (11–13) As the number of potential applications for optical imaging grows, so does the number of tracer designs that provide the optical contrast. Though untargeted dye solutions had been in use for quite some time already, optical tracers based on antibodies were among the first targeted probes to show feasibility *in vivo*. A diverse array of alternative imaging scaffolds has since been developed, like single-chain antibodies and other nanobodies (14–19), small peptides (20–22), targeted aptamers (23), small molecules functioning as either dye carrier (24,25), substrates for endogenous fluorophore generation (26,27) or activatable probes (28), quantum dots (28), or reactive micelles (29,30). Despite these innovations, conjugated antibodies have remained a mainstay of the tracer arsenal. It should be noted that there is significant difference between all above mentioned imaging modalities, and each of their individual properties should be carefully considered in relation to the envisioned application of the probe. In addition, most of these modalities are still in the preclinical testing stage, and only antibodies and nanobodies have reached clinical trials. Due to the complexity and the number of factors to take into consideration, it is not possible to declare one modality as unequivocally "better" than the others, and rather than viewing the different forms of optical imaging as competitive, they should be seen as complementary. For instance, antibodies display slow tissue distribution and a long (plasma) half-life for circulation and clearance. Studies with PET-labelled antibodies have shown that the tumour to background ratio increases over time as the tracer is cleared from circulation. (31,32) For this reason, trials investigating conjugated antibodies typically wait several days (1-7, typically 2-3 (4–6,10,33–38)) between the intravenous injection of the tracer and imaging of the response. In cases where this is logistically unfeasible, a lower molecular weight tracer with more rapid distribution and shorter half-life may be used. However, while a lower molecular weight tracer will achieve peak levels in tissue faster, it is also cleared faster, resulting in less time to accumulate in tissue of interest, and reduced retention of the signal as the tracer is eliminated. This rapid elimination can be problematic if the imaging moment takes place much later than planned, as this could result in the tracer washing out of the tissue and signal becoming less detectable. Antibodies, thanks to their long half-life, are likely to remain detectable hours to days later. To achieve similarly long windows for exposure, doses of peptide or small molecule tracers will have to be much higher than for antibodies (relatively), which could result in more toxicity. This is just one illustration of how every indication for optical imaging should be matched to a tracer that either fits within or can be adapted to existing

clinical procedures, in order to provide the most benefit for both patient and healthcare practitioner. Additionally, it may be possible to combine different tracers to improve contrast and broaden the applicability of the technique, as we also showed for the PD-1/PD-L1 tracer pair in chapter 7. Similar approaches to the complementary antibodies have been investigated, for instance through combining a specific targeted antibody with an aspecific antibody (like polyclonal IgG), or combining a specifically targeted nanobody with dye lacking any specific binding. The same approach may well work when combining specific tracers based on different molecular designs. (39)

The initial ideas around optical imaging within oncology was to apply it as a red-flag technique to demarcate tumours and assist the surgeon in distinguishing tumour from healthy tissue during Fluorescent Guided Surgery (FGS). It has been shown in several studies that tumour-specific fluorescent signal can be correlated both with tumour tissue and with the distribution of the targeted antigen on histology. (4,34,37,40,41) However, in practice the useability of in-situ delineation of tumour tissue varies, and depends on the type of tracer used, the morphological position and surrounding tissue of the tumour, and the extent to which this allows for homogenous illumination and viewing of the tumour. Therefore, a more valuable role for optical imaging may be in the flagging of incomplete resections, close or positive resection margins, small local metastases and positive lymph nodes in the direct environment of the main tumour during surgery (1,34,42). In these situations, the surgeon would use the fluorescence from a previously administered tracer during an add-on procedure after routine visual inspection and palpation, rather than relying purely on the fluorescence to perform the resection. These fluorescence-enhanced inspections can take place in or around the wound bed, but may be just as valuable to perform on the basal side of a resected tissue sample, where any signal that "breaks through" could indicate a margin that is close or positive, as a result of the 8-10 mm tissue penetration of near-infrared light. Such a break-through signal could then be used as an incentive for additional tissue resection, or investigation of the positive spot by frozen section biopsy.

As an alternative, or addition, to the FGS approach, fluorescence could also be employed during pathological tissue processing. Scanning of the basal side of the specimen could be seen as a form of performing fluorescence guided pathology (FGP). Due to processes such as formalin fixation and paraffin embedding, tissue sectioning, staining and microscopic inspection, traditional pathologic reports have a considerable lag time between the invasive procedure and the result (1,42,43). Similar to the procedures described above for FGS, scanning resected tissue, lymph nodes or biopsies for fluorescence generated by a previously administered tracer can guide the pathologist to choose which parts of the resected tissue should be microscopically investigated for presence of disease. This could reduce both the number of missed positive results as well as reduce the number of overall samplings, as negative samples can be ruled

out prematurely. (44) Alternatively, it may be possible to apply a tracer *ex vivo* on the tissue and achieve the same effect in an even shorter time span, making it feasible to provide feedback to the surgeon directly. This may even work with the antibody tracers described in this thesis which, despite their typically slow distribution and pharmacokinetics, were able to visualize dysplasia and cancer 5 minutes after topical application, during endoscopy for Barret's esophagus, without the need for long distribution or incubation time. (6) Another approach to FGP could be possible *in vivo*. Since an optical tracer can "tag" tissue *in vivo*, using mesoscopic or microscopic imaging techniques during the invasive procedure can allow cell-level investigations in patients, essentially enabling *in vivo* immunohistochemistry or optical biopsy. This may enable targeted biopsy or instant investigation of a resection wound bed to estimate the negativity of the margin before confirmation with standard-of-care pathology. A proof of concept of this approach was already shown using two different conjugated antibodies for inflammatory bowel disease (adalimumab and vedolizumab), where the FITC-labelled protein was sprayed on colon wall and investigated with confocal laser endomicroscopy (11,12). Labelled cells were visible and could be counted, and a correlation between the number of cells and biological therapy outcome could be shown.

These fluorescent molecular imaging studies in IBD using topical FITC-labelled antibodies also highlight a new transition that is currently being made in the imaging field. So far, the bulk of optical imaging trials have been performed in oncology, but recently studies have begun to investigate the application of optical imaging in inflammatory bowel disease (IBD). The potential applications for IBD are quite different as in general the endoscopist does not require extra contrast to determine the location or severity of inflamed tissue or ulcerations, as these are clearly distinguishable by standard-of-care high-definition white-light endoscopy (HD-WLE), or additional techniques like narrow-band imaging. (45,46) In this situation, optical imaging would not be used for lesion localization, but rather for targeted biopsy, to visualize distribution of a labelled drug in the mucosa of the digestive tract, to highlight specific populations of immune cells within tissue, or to visualize intra-patient heterogeneity. In addition, when using therapeutic biologicals licenced for inflammatory diseases as tracer, or a similar ligand that can show availability of a therapeutic target, it may be possible to predict response to biologic therapy at the start of therapy, rather than waiting for clinical response evaluation, which could take several weeks. (47) Imaging techniques can also be used to visualize the degree of target saturation, and thereby be used in the evaluation and personalization of drug dosing, based on the deduced local concentrations.

FUTURE PERSPECTIVES

Many studies have already shown the feasibility and visibility of optical tracers for delineation and localization of lesions both *in vivo* and *ex vivo* in clinical trials. A next step in the development for optical imaging technology would be to move upward from these first studies that only show the visualization of molecular targets, and progress to studies that relate imaging outcomes to patient benefit. Building upon the dose-finding studies performed so far, prospective investigations into the predictive properties and diagnostic accuracy of tracer procedures should be performed. Ideally, this trial will involve randomization of patients across a group that receives an invasive procedure with optical imaging, and one without an optical component that receives the same treatment otherwise. Both groups can then be compared on primary outcomes or surrogate markers. A small number of phase 3 studies on the use of a fluorescent marker during surgery (SGM-101 and OTL38) have been performed or are ongoing, and this example should be followed by more investigators in order to lay a solid foundation for incorporation of the technology into clinical practice, and to allow comparison between different applied forms of the technique.

One topic that still requires more elaboration for proper design and standardization of trials for optical tracers is the approach towards blinding of the clinicians and investigators. In a trial with a randomized double-blind design, the preferred setup would be for both the patient, the treating physicians and the investigators to be blinded to what treatment arm a patient is in, to avoid bias in the evaluation of the method. By design, the use of tracers is visible, and therefore once the camera is turned on, it would be very easy to find out if a patient was randomized to a treatment arm or a placebo arm. Careful consideration should be given to what the impact of this inherent unblinding would be, and if there are additional ways to objectively assess the impact of using a tracer-guided resection or endoscopy. For instance, a sham fluorescent imaging procedure may be considered for the placebo group patients. This would make sure that all patients receive an additional visual inspection based on fluorescence camera footage, to make sure treatment arm patients don't show more complete resection purely due to longer time spent inspecting the surgical field. Alternatively, incorporation of cross-over designs and multiple (randomized) treating endoscopists to perform the procedure may reduce the risk of bias. This approach normalizes the number and types of procedure a patient undergoes, and introduces multiple independent observers to improve the feasibility of extrapolating the study results to the general practice. Another topic in this same field is the use of placebo fluorophore. Due to their fluorescent nature tracers are typically coloured quite noticeably in solution, and if the placebo copies the colour of the solution, the design of a placebo solution would have to incorporate a check of fluorescent properties. Any dye added to the placebo cannot have fluorescent properties that may be picked up by camera, as this may severely impact the results of the placebo procedures, especially once interventions can be made based on tracer signal.

Within the context of these outcome-focused trials, many of the practical considerations about different tracer forms mentioned previously (antibodies, nanobodies, smaller molecules) may be utilized to find new promising applications for optical imaging and allow the selection or design of a tracer that is suitable for this procedure, rather than adjusting the procedure to the tracer properties. Each procedure for which optical imaging is considered has a different overall schedule for patient and healthcare practitioner, as well as different limits and requirements with regard to overall procedure time, potential to compensate for delays, and preparation time before the procedure, which may influence when the logistically optimal time for tracer administration is. Taking these procedure-specific requirements into account, trial design could be based around fitting in with existing procedures and timeframes first, rather than defining study parameters around the limitations of the tracer. For example, tracer administration may be performed at a "sub-optimal" time point if this means an easier incorporation of tracer imaging into routine care, as long as the acquired data and the decisions stemming from those data are not influenced significantly.

A more novel aspect of optical imaging that still has potential to grow is the application of optical imaging for drug development. PET and SPECT labelling have both been used for a multitude of drugs to gain insight into drug pharmacokinetics and -dynamics, and to show distribution on a macroscopic scale by highlighting organs that demonstrate uptake of the radioactive drug. (48,49) Optical imaging could play a similar role, but on the microscopic scale. Within a target organ, wide-field imaging using optical probes could provide insight into the distribution of the drug in specific parts of the organ, on a resolution that is not feasible for radioactive probes. In addition, information about drug distribution on a cell-level and the mechanism of action can be generated by imaging tissue biopsies. Fluorescent tracers have been shown to still be detectable in biopsy material after intravenous administration in the patient. (34,43) Combining this tracer-based fluorescent signal from the tissue with other markers for fluorescence microscopy that are applied to the tissue externally can provide insight into the molecular interactions of the tracer. In addition, optical imaging can highlight within-patient heterogeneity. By tracking where in the body the conjugated drugs are located on a small scale, patches of tissue that are fluorescent could be compared to tissue that is not fluorescent, and the reason for (lack of) effect can be investigated.

To strengthen the association between tracer distribution and observed effects, technologies like multi-diameter single fiber reflectance single fiber fluorescence (MDSFR/SFF) spectroscopy can be used to measure a fluorescent signal and correct for tissue optical properties and reflectance of the excitation light. This provides a more standardized, quantitative value than can be attained by any camera-based modality at this point. (50,51) Further developments in this technique could allow real-time quantitative assessment of drug distribution, and provide insight into local drug concentrations in the tissue of interest, which is currently lacking for many products.

PHARMACEUTICAL DEVELOPMENT OF NEAR-INFRARED TRACERS

This thesis describes several aspects of the pharmaceutical development and design of the tracer molecule that are key for application of optical imaging in the clinic. This is a discipline that, despite its importance for performing optical imaging procedures, is often underrepresented in scientific literature. Discovery of imaging modalities, and the first synthesis of novel compounds may be published, but only very limited data are available for the phase between the laboratory experiments and the clinical trial. Based on the contents of this thesis, we give two recommendations with regard to clinical translation of novel products. First, pharmaceutical design should be considered as early as possible to prevent major delays down the road, and should be in an advanced state by the time the clinical trial application is submitted. One part of clinical translation is assessing the quality of all input materials and process aids. During this assessment, starting materials, raw materials, and process aids are identified, evaluated and exchanged for a material of Ph.Eur. grade or similar clinical grade, to be fit for human application. Where no clinical grade materials are available, a material of the highest grade available should be used. It should be noted that change of starting materials, especially in biotechnological or advanced therapy medicinal product (ATMP) production processes, may lead to a different result of the production, and should be considered a critical change to the process. Such a change will have to be assessed and reviewed in depth, and the potential impact on the product should be evaluated, justified and documented before the implementation of the change may be completed. By incorporating pharmaceutical design in the early parts of the development (if possible as early as the first proof of concept studies), problems in translation down the line can be avoided, and early development can already contribute to the later clinical translation.

Second, more in-depth investigation into the pharmacokinetic and pharmacodynamic actions of tracers should be performed. This is especially true for tracers based on licenced drug products. For both radioisotope- and optical dye-labelled antibodies, conjugation techniques based on n-hydroxy-succinimidyl (NHS) esters are frequently employed, which results in a random distribution of the labelled compound on the targeting scaffold. As we showed in chapter 8, conjugating dyes can have different effects per antibody and per dye. While there is a common assumption that pharmacokinetic parameters of tracers are unchanged from (or at least highly similar to) their originator molecule, evidence for this is limited, and studies that did investigate it show half-lifes of fluorescently labelled antibody in human subjects to be different from the reported half-life in the summary of product characteristics for the clinically approved antibody. (36,38,52,53) In addition, the effect of dye conjugation as observed in PBS or similar solutions may not be comparable to the performance of tracer under biological conditions, as a result of post-administration modifications. (54) Pharmacokinetics of the antibody tracer are not only based on the properties of the molecule,

but may also be influenced by the tracer dose that was administered. The results of an accurate pharmacokinetic and -dynamic assessment can assist decision-making for the settings and conditions in which the final product is applied, and should be incorporated in the design of advanced studies to determine predictive accuracy as mentioned before.

Optical imaging as a whole is on the cusp of transitioning into a new phase, with technologies for both wide-field detection and *in vivo* quantification being refined, the stage is set for trials that show the value of the technique. One antibody-based tracer and one small molecule have been tested in phase III (SGM-101: NCT03659448, NCT04642924, earlier results were published: (9,10,55); OTL38: NCT03180307, NCT04241315) which could provide a precedent for how the technique can be applied in routine clinical care. More tracers and devices should go this route in order to create a foothold for the technology to build on, though care should be taken not to introduce multiple similar, but still distinct techniques in parallel before methods for comparability have been investigated. The introduction of multiple distinct imaging platforms (potentially each with their own tracer product) carries the risk of dividing the community into clusters based around specific imaging systems, as well as muddying the waters for new groups who may have difficulty picking the system to use for their experiments. Like with nuclear imaging, optical imaging research should strive for the quantified fluorescence signals to be expressed in objective units that can be compared between dyes, tracers, imaging devices and treatment centres, similar to the standardized uptake values (SUV) as used for PET. Fortunately, this aspect is already being tackled by exploration of the possibilities for establishing imaging phantoms for device performance comparison and calibration, and the formation of a task group for fluorescence guided surgery based out of the American Association of Physicists in Medicine. (56–58) This can form the basis for proper comparison of methods and results, and the focused development of optimal tracers and imaging systems for many applications. The MDSFR/SFF spectroscopic measurements mentioned previously could also be a way to get to objective fluorescent intensity values, that can be easily compared to measurements in other centres.

MAIN CONCLUSIONS

Many interesting potential applications for optical imaging can be imagined both within surgery, endoscopy and pathology, and the technology is getting to the level required to perform imaging in a way fit for clinical care. If the community makes an effort to strive towards outcome-focused trials and the standardization of signal detection and evaluation, implementation of optical imaging in invasive procedures or during pathological assessment of tissue could be achieved in the near future, when phase III trials have been completed. The adoption of this modality can be of great value for highlighting affected tissue, guiding biopsies, streamlining pathology and visualizing drug distribution, which could reduce surgical miss rates, reduce workload for pathological tissue assessment, and increase insight into the mechanism of new drugs. Now that both near-infrared tracers and detection modalities are growing out of the experimental phase and are moving towards clinical integration, there are major opportunities for this modality on the horizon, and the future looks bright for fluorescent optical imaging.

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