CHAPTER 7

Summary, general discussion and future perspectives
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SUMMARY
Current cancer treatment strategies consist of surgery, radiotherapy and systemic treatment. During the past two decades, molecularly targeted therapy and cancer immunotherapy have greatly expanded cancer treatment options next to classic chemotherapeutic drugs, such as taxanes and platinum-based anticancer drugs. Targeted therapies aim to inhibit molecular pathways important for tumor growth and survival, whereas cancer immunotherapy stimulates a host immune response to induce tumor cell kill. One of the first known molecular targets has been the extensively studied human epidermal growth factor receptor (HER) family of receptor tyrosine kinases. Drugs that block HER family members (epidermal growth factor receptor (EGFR) and HER2) or inhibit downstream signaling by HER family members, including human anti-HER antibodies and tyrosine kinase inhibitors (TKIs), are increasingly becoming available. However, intrinsic or acquired resistance against HER-targeted agents is a clinical problem, and much research focuses on optimizing targeting of the HER family (1,2).

Rapid non-invasive assessment of changes in HER protein expression as a result of treatment with HER-targeted agents would be of major interest for clinical decision making. Non-invasive molecular imaging, could potentially be used as a tool to monitor these membrane receptor dynamics. Molecular characteristics, such as HER cell surface expression, can be imaged with three-dimensional whole body positron emission tomography (PET) using antibodies radiolabeled with zirconium-89 (89Zr). HER antibody based PET-imaging could play an important role in identifying patients who might benefit from anti-HER antibody therapy, as well as monitoring responses to HER-targeting agents. Programmed cell death 1 (PD-1)/ programmed death-ligand 1 (PD-L1) checkpoint blockade with antibodies, which are examples of cancer immunotherapy, has resulted in significant long lasting responses in patients with advanced-stage cancers (3–5). Recently, it has been shown that EGFR plays a role in PD-L1 expression by tumor cells (6,7). EGFR inhibition reduces PD-L1 expression in vitro, resulting in a concomitant increase in T cell activation. Therefore, EGFR inhibition may be used to improve efficacy of immunotherapy.

Taken together, insight in cellular plasma membrane protein dynamics might well be important for proper designing (combination) therapies. Therefore, this thesis aimed to study the effect of HER-targeting agents on HER and PD-L1 dynamics to provide a rationale for future combination therapies involving therapies targeting the HER-pathway and immune checkpoints. In addition, HER tracers for molecular imaging to evaluate drug tumor targeting, organ distribution and target dynamics were explored. Combining two non-competing anti-EGFR monoclonal antibodies can enhance downregulation of the EGFR-signaling pathway, and showed superior anticancer efficacy.
compared with single anti-EGFR antibody treatments in several human tumor xenograft models (8–10). Cetuximab and imgatuzumab are monoclonal antibodies directed against distinct, non-overlapping epitopes in EGFR extracellular domain III. Moreover, imgatuzumab is a glycoengineered antibody to enhance antibody-dependent cellular cytotoxicity (ADCC) responses (11). Thus, the combination of both antibodies is a potential strategy to target EGFR more effectively than existing treatments with single antibodies. Therefore, in chapter 2, we investigated anti-EGFR monoclonal antibodies imgatuzumab and cetuximab–induced internalization and membranous turnover of EGFR, and whether this affected imgatuzumab–mediated ADCC responses and growth inhibition of non-small cell lung cancer (NSCLC) cells. In a panel of wild-type EGFR expressing human NSCLC cell lines, membranous and total EGFR levels were downregulated more effectively by imgatuzumab when compared with cetuximab. Imgatuzumab plus cetuximab enhanced EGFR internalization and reduced membranous turnover of EGFR, resulting in an even stronger downregulation of EGFR. Immunofluorescent analysis showed that combined treatment increased clustering of receptor-antibody complexes and directed internalized EGFR to lysosomes. The antibody combination potently inhibited intracellular signaling and epidermal growth factor (EGF)-dependent cell proliferation. More importantly, robust EGFR downregulation after treatment for 72 hours with the antibody combination did not impair ADCC responses. In conclusion, imgatuzumab plus cetuximab lead to a strong downregulation of EGFR and superior cell growth inhibition in vitro without affecting antibody-induced ADCC responses.

Whole body determination of EGFR expression in lesions using PET imaging could support decision making during clinical development and clinical practice. Preclinical EGFR-microPET imaging revealed a mismatch between EGFR expression in tumor lysates assessed with Western blotting and in vivo tumor uptake of the radiolabeled EGFR antibody tracer assessed with PET (12). Shed EGFR ectodomain, which is present in cancer patient sera, can potentially bind tracer and therefore affect tracer kinetics (13). In chapter 3, we examined the influence of shed EGFR levels in the blood stream on tracer kinetics and tumor uptake of EGFR monoclonal antibody $^{89}$Zr-imgatuzumab in varying xenograft models in order to optimize $^{89}$Zr-imgatuzumab-PET. Human A431 (EGFR overexpressing, epidermoid carcinoma of the vulva), A549 and H441 (both EGFR medium expressing, NSCLC) cancer cell lines were xenografted in mice. Tumor bearing mice received various doses of $^{89}$Zr-imgatuzumab, co-injected with equal doses of $^{111}$In-IgG control. $^{89}$Zr-imgatuzumab uptake in A431 tumors was highest (29.8 ± 5.4 %ID/g) in the 160 μg dose group. Contrary, highest uptake in A549 and H441 tumors was found at the lowest $^{89}$Zr-imgatuzumab dose (10 μg). High $^{89}$Zr-imgatuzumab liver accumulation was found in A431 xenografted mice, which decreased with antibody dose increments. $^{89}$Zr-imgatuzumab liver uptake in A549 and H441 xenografted mice was low at all doses.
Shed EGFR levels in liver and plasma of A431 bearing mice were up to 1000-fold higher than levels found in A549, H441 and non-tumor xenografted mice. In conclusion, $^{89}$Zr-imigatuzumab effectively visualized EGFR-expressing tumors. High shed EGFR levels redirected $^{89}$Zr-imigatuzumab to the liver, in which case tumor visualization can be improved by increasing tracer antibody dose.

HER3 is the only member of the HER family lacking intrinsic tyrosine kinase activity and therefore its role in cancer has long been underestimated. However, in view of its overexpression in various tumor types, and compensatory role in HER signaling, HER3 has gained much interest as a potential target in cancer treatment. In chapter 4, a review describes the biology and relevance of HER3 in cancer, as well as drugs that block HER3 or interfere with HER3 dimer signaling. These drugs include fully human anti-HER3 antibodies, bispecific antibodies and TKIs, and are currently becoming available for clinical use. An overview of HER3-targeting drugs in clinical trials is given. Biomarkers might be useful for prediction and monitoring of treatment effects, as well as supporting decision making during clinical development and clinical practice. Therefore, potential biomarkers for effective HER3-targeting such as tumor analysis of HER3 expression, functional assays for downstream effector molecules and molecular imaging techniques are discussed.

HER3 membrane expression is very dynamic due to its compensatory role in HER signaling, particularly in response to drugs targeting HER family members or the phosphoinositide 3-kinase (PI3K) pathway, which may attenuate the antitumor action of these inhibitors (1,14–16). Preclinically, combining HER3 with HER2 inhibitors augmented tumor growth inhibition, in vivo (17). This might advocate for a more dynamic, preferably non-invasive assessment of HER3 tumor status than immunohistochemistry. Molecular imaging techniques can potentially address this. Therefore, in chapter 5, we explored in vivo HER3 tumor status assessment after lapatinib (dual EGFR/HER2 TKI) treatment with $^{89}$Zr-labeled anti-HER3 antibody mAb3481 PET. In vitro, lapatinib increased membranous HER3 in BT474, SKBR3 and N87 cells, and consequently increased mAb3481 internalization by 1.7-fold (BT474), 1.4-fold (SKBR3) and 1.4-fold (N87). $^{89}$Zr-mAb3481 BT474 tumor uptake was remarkably high at SUV$_{\text{mean}}$ 5.6±0.6 (51.8±7.7 %ID/g) using a 10 µg $^{89}$Zr-mAb3481 protein dose in vehicle-treated mice. However, compared to vehicle, lapatinib did not affect $^{89}$Zr-mAb3481 ex vivo uptake in BT474 and N87 tumors, while HER3 tumor expression remained unchanged. In conclusion, lapatinib increased in vitro HER3 tumor cell expression, but not when these cells were xenografted. $^{89}$Zr-mAb3481 PET accurately reflected HER3 tumor status. $^{89}$Zr-mAb3481 PET showed high, HER3-specific tumor uptake, and such an approach might sensitively assess HER3 tumor heterogeneity and treatment response in patients.
Recently, it was shown that constitutively activated mutant EGFR induces PD-L1 expression in NSCLC cell lines (7). EGFR inhibition reduces PD-L1 expression of these EGFR mutant cell lines, resulting in a concomitant increase in T cell activation. However, in the patient setting, EGFR mutant NSCLC tumors do not respond to PD-1 blockade, even when PD-L1 expression is high. This is due to several factors such as lack of CD8+ T cell infiltration and low tumor mutational burden (18). In contrast, PD-1 blockade has improved survival of EGFR wild-type NSCLC patients (18–20). Surprisingly, there is only limited data about the regulation of PD-L1 expression in EGFR wild-type NSCLC (21). Improved understanding of PD-L1 regulation may provide a rationale to combine immune checkpoint inhibitors with other targeted agents. Therefore, in chapter 6, we aimed to identify pathways regulating PD-L1 expression in EGFR wild-type NSCLC by using RNA-seq data from The Cancer Genome Atlas (TCGA) lung adenocarcinoma and squamous cell lung carcinoma data sets. Analysis of RNA-seq data of EGFR wild-type NSCLC tumors revealed that inferred interferon gamma (IFNγ) and mitogen activated protein kinase (MAPK) signaling correlated with PD-L1 gene expression in lung adenocarcinoma. In EGFR wild-type lung adenocarcinoma cell lines, stimulation with EGF or IFNγ strongly increased PD-L1 mRNA, protein, and membrane expression, which was further enhanced by combining EGF and IFNγ. Similarly, tumor cell PD-L1 membrane expression increased after coculture of activated peripheral blood mononuclear cells (PBMCs). Inhibition of the MAPK pathway, using EGFR-inhibitors cetuximab and erlotinib or mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) inhibitor selumetinib, prevented EGF and IFNγ-induced PD-L1 mRNA, protein, and membrane upregulation, but had no effect on IFNγ-induced major histocompatibility complex (MHC) class I upregulation. Interestingly, while IFNγ increases transcriptional activity of PD-L1, MAPK signaling acted both through increased transcription and stabilization of PD-L1 mRNA. In conclusion, MAPK signaling plays a key role in EGF and IFNγ-induced PD-L1 expression in EGFR wild-type adenocarcinoma and may present a target to improve efficacy of immunotherapy.

In conclusion, this thesis provides new leads for future combination therapies involving drugs targeting the HER-pathway and immune checkpoints. Furthermore, new PET imaging tracers have been developed to assess tumor targeting and organ distribution, as well as monitoring HER family dynamics.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Improving antibody based EGFR-targeted therapies
Over the past decades, it has become evident that HER family members have a prominent role in the development of several frequently occurring types of solid tumors. This has led to the development and widespread implementation of specific HER-targeted
therapies. Although these therapies have provided substantial benefit to patients with advanced cancer, resistance to these therapies often develop. This underscores the need for new treatments to improve survival.

Having insight in HER protein dynamics as a result of treatment with HER-targeted agents would be of major interest for designing (combination) therapies that target the HER family. By studying the dynamics of EGFR we show that in vitro the combination of imgatuzumab and cetuximab led to a strong downregulation of EGFR and potently inhibited downstream signaling and cell growth (Chapter 2). Interestingly, we and others have shown that anti-EGFR antibodies, including imgatuzumab and cetuximab, can increase EGFR auto-phosphorylation, but this had no effect on downstream PI3K/Akt or MAPK signaling. Whether EGFR phosphorylation activates other downstream pathways of EGFR that have an effect on tumor cells, is still unknown (23). Noteworthy, the combination of imgatuzumab and cetuximab counteracted the increase in EGFR auto-phosphorylation levels observed after single antibody treatment. Therefore, combining EGFR antibodies might be the preferred strategy to inhibit EGFR signaling. Complete inhibition of EGFR may still not be sufficient since intrinsic or acquired resistance to targeted therapies can occur through e.g. on-target mutations or compensating activation of alternative pathways. However, monoclonal antibody therapies can also have anti-tumor effects by modulating immune responses. Since ADCC is thought to be an important mechanism of certain monoclonal antibodies, several techniques are currently being employed to enhance ADCC capabilities. This can for example be achieved using defucosylation techniques, as was done for imgatuzumab. Imgatuzumab showed superior in vivo efficacy compared with cetuximab or the non-glycoengineered variant of imgatuzumab in a series of mouse xenograft models, suggesting that ADCC contributes to the anti-tumor response. By studying the effects of anti-EGFR antibodies on EGFR dynamics, we showed that long-term treatment with imgatuzumab with or without cetuximab massively targets EGFR to the lysosomal degradation pathway. Although loss of EGFR surface expression might suggest impairment of imgatuzumab-dependent ADCC responses, our data show that ADCC responses are actually not reduced in a panel of low to moderate EGFR expressing NSCLC cell lines. These results indicate that the threshold to induce the maximum amount of ADCC in this experimental setting is still maintained. A recent study revealed a decrease in peripheral natural killer cells in patients with head and neck squamous cell carcinoma treated with imgatuzumab but not when treated with cetuximab, suggesting imgatuzumab mediated accumulation of NK cells via FcγRIIIA in EGFR expressing tissues. In addition, equal efficacy of imgatuzumab and cetuximab in terms of EGFR pathway inhibition were found (23). Unfortunately, further clinical development of imgatuzumab was stopped after it failed to meet its primary endpoint in a phase II trial in patients with colorectal cancer. On the contrary, glycoengineered
antibody treatment has already been successfully implemented for hematological tumors. Obinutuzumab, an anti-CD20 antibody with enhanced ADCC function for the treatment of chronic lymphocytic leukemia showed superior progression free survival over its non-glycoengineered predecessor rituximab. Whether glycoengineering of the antibody was the main contributor to this enhanced ADCC effect remains unknown, since obinutuzumab and rituximab differ in other aspects as well, e.g. binding to different epitopes. Despite the fact that evidence is lacking whether enhanced ADCC improves anti-tumor responses in patients, there is still a great interest in increasing ADCC by modifying antibodies or using combination treatments. For example, it was shown in human xenograft models that targeting the costimulatory molecule CD137 on natural killer cells with an agonistic monoclonal antibody enhanced ADCC activity of cetuximab (24). A recent study showed that coadministration of imagatuzumab with cytotoxic agents enhances in vivo efficacy in various xenograft models. The ADCC activity of imagatuzumab was not impaired by conventional cancer premedication drugs and chemotherapy (25). In addition to eliciting ADCC responses, IgG1 monoclonal antibodies, including cetuximab, can also induce CD8+ T effector cell-dependent antitumor activity in human xenograft models (26). In patients with head and neck squamous cell carcinoma treated with imagatuzumab or cetuximab, a trend toward increased numbers of tumor infiltrating T cells was observed (23). Thus, anti-EGFR monoclonal antibodies might synergize with T-cell activation, which can be further enhanced by blockade of immune checkpoints such as the PD-1/PD-L1 interaction. Since anti-EGFR antibody combinations (Chapter 2) showed superior preclinical activity, it will be of interest to investigate mixtures of anti-EGFR antibodies in treatment strategies using chemotherapy and immunotherapy.

Another approach to target HER receptors, but bypass resistance to downstream signaling inhibition, is the use of antibody-drug conjugates (ADCs) that deliver toxic payloads to tumor cells via antibody-receptor interactions. ADCs combine targeting properties of monoclonal antibodies with the antitumor effects of potent cytotoxic drugs, such as microtubule inhibitors. ADCs need to be internalized by the cell and, for most ADCs, subsequently transported to the lysosome where the drug is released and can exert its cytotoxic effects on tumor cells, stressing the importance of studying plasma membrane protein dynamics. A limitation of EGFR-targeted ADCs is the significant toxicity risk due to EGFR expression in normal tissue. Therefore, an anti-EGFR ADC that targets a unique tumor-specific EGFR epitope in the CR1 extracellular subdomain, which is only accessible in tumors expressing the deletion mutant EGFR variant III or overexpressing wild-type EGFR, is currently being developed (27). The results from this thesis provide a rational to combine a tumor specific anti-EGFR ADC with another non-competitive anti-EGFR monoclonal antibody that targets the same subdomain in order to enhance internalization and lysosomal localization of the ADC. Alternatively, HER3 can be used...
as a target for ADCs, since HER3 expression is strongly increased after HER2, HER3 and PI3K pathway inhibition. Interestingly, HER3-antibody complexes are even more rapidly internalized as compared to EGFR-antibody complexes, which may be advantageous for ADC activity (Chapter 5).

**Immuno-PET of HER proteins with $^{89}$Zr-Labeled Monoclonal Antibodies**

Immuno-PET of HER proteins has the potential of expanding our knowledge of whole-body target expression, biodistribution, tumor delivery and accessibility for antibody-based targeted therapies. This might be useful for prediction and monitoring of treatment effects of HER-targeted therapies, as well as supporting decision making during clinical development and clinical practice. So far one study was designed to interrogate the role of $^{89}$Zr trastuzumab-uptake in relation to treatment effect. Lack of uptake of $^{89}$Zr-trastuzumab in tumor lesions indeed coincided in these patients with a shorter time to treatment failure to the ADC trastuzumab-emtansine in HER2-positive breast cancer (28). Whether immuno-PET of EGFR can be implemented for prediction and monitoring of treatment effects is unknown. A substantial hurdle is that preclinical EGFR antibody-based imaging studies revealed a mismatch between *in vivo* EGFR protein expression and tumor tracer uptake (12). This could be influenced by many factors including perfusion rates, vascularity, vascular permeability, interstitial pressure and antibody plasma half-life, but also EGFR protein dynamics. In chapter 3, we show that shedding of the EGFR extracellular domain is another factor that influences tumor targeting of this antibody. These findings indicate that shed EGFR levels have to be taken into account in the assessment of preclinical and probably clinical EGFR imaging. Extracellular domain shedding may also present diagnostic problems for the molecular imaging of other antigens, such as HER2. Similar to many other *in vivo* studies with anti-human antibody-based imaging tracers our study has some species-specificity limitations, since imgatuzumab only binds to human shed EGFR and lacks cross-reactivity with murine shed EGFR. The use of antibodies that bind to both the human and mouse target protein can provide additional information about the influence of endogenous antigens on tracer kinetics. In addition, most immuno-PET studies, including our study, have only focused on the antigen-binding fragments (Fab) of monoclonal antibodies that confer binding specificity. However, full-length antibodies also contain an Fc region that bridges antibody-coated targets with Fcγ receptor (FcγR) positive immune cells. For example, tumor-associated macrophages are able to capture antibodies via their FcγRs and thus further complicate the relationship between tracer uptake and target protein expression (29). Future preclinical diagnostic efforts to image HER protein expression should consider human immune system engraftment models to study these interactions or develop imaging agents incapable of binding to FcγRs.
Monitoring treatment effects on HER family dynamics using immuno-PET

This thesis aims to monitor treatment effects on HER family dynamics using immuno-PET as a potential biomarker for effect of HER-targeting therapies. Unfortunately, using $^{89}$Zr-labeled anti-HER3 antibody mAb3481 PET, the increased HER3 expression on tumor cells after in vitro lapatinib treatment was not observed in vivo using tumor bearing mice (Chapter 5). A drawback of our study might be the narrow therapeutic window of lapatinib to upregulate tumor HER3 expression in vivo without having major effects on tumor growth. The feasibility of detecting variations in HER3 expression can be further explored preclinically using stably transfected cell lines overexpressing different HER3 cell surface levels instead of using different cell lines with various HER3 levels. These isogeneic models may strongly reduce complicating factors, such as changes in tumor growth, perfusion rates and vascularity. In this thesis, only full size antibodies are used for PET imaging. Smaller proteins such as affibodies or antibody fragments may be better options for monitoring treatment effects on HER family dynamics. Major advantages of their smaller size are the rapid tumor penetration and the fast clearance from the blood and nonspecific compartments. This makes it possible to perform PET imaging in a shorter timeframe and allows for repeated scans pre-, during- and post-treatment. The assessment of HER signaling by quantifying and visualizing phosphorylation of active sites in HER proteins or downstream molecules might further contribute to insight in which patients benefit from HER-targeting therapies. This especially applies to therapies that are designed to inhibit receptor signaling. However, this would require molecules that can pass the cell membrane. Currently, antibody-based and small molecule tracers that can visualize intracellular and intranuclear proteins both in vitro and in vivo are being developed preclinically (30,31). An alternative strategy to monitor early treatment response to HER-targeted therapies can be to measure changes in dynamics of other proteins excreted or expressed extracellularly using immuno-PET. In this case, these proteins would serve as “effect sensors” of a treatment (32).

Effect of HER-targeted therapies on dynamics of immune checkpoints

Immune checkpoint blockade is a relevant therapy across several tumor types, including NSCLC. Although anti-PD-1/L1 therapy has already shown efficacy in EGFR wild-type NSCLC, many patients still do not respond. Combination treatments are needed to further increase the efficacy of immune checkpoint blockade.

In chapter 6, we show that EGFR pathway inhibition using EGFR-targeted therapies cetuximab and erlotinib, or the MEK inhibitor selumetinib strongly reduced EGF- and IFNγ-dependent PD-L1 expression in EGFR wild-type NSCLC cell lines. Lowering PD-L1 expression using EGFR pathway inhibitors combined with other checkpoint inhibitors, such as PD-1 or CTLA-4 blocking antibodies might be a strategy to increase efficacy in
NSCLC. A drawback of our study is the lack of functional assays for measuring the effect of PD-L1 downregulation on T cell function. Recent data using the CT26 colon cancer syngeneic mouse model support our concept (33). This study demonstrated that even though MEK-inhibition reduces anti-tumor priming of T cells in lymph nodes, it potentiates the anti-tumor immune response by preventing apoptosis of tumor infiltrating T cells. Combined with anti-PD-L1 treatment this resulted in sustained tumor regression (33). In NSCLC, several phase I clinical trials are already ongoing, combining MEK or EGFR inhibitors with immunotherapies such as anti-CTLA4, anti-PD1 and anti–PD-L1. Their results are eagerly awaited. In addition, interest in targeting novel immune checkpoint receptors is increasing. Recent studies have shown that the co-inhibitory receptors T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) and Lymphocyte-activation gene 3 (Lag-3) are potential targets for immune checkpoint inhibition in NSCLC (34). Blocking antibodies against Tim-3 or Lag-3 alone or in combination with anti-PD1 antibodies are undergoing phase I testing in advanced or metastatic solid tumors. Whether HER-targeted therapies affect the dynamics of such immune checkpoints is still unknown.

This thesis focusses on immuno-PET of HER proteins. However, it will also be of interest to investigate whether changes in PD-L1 expression levels, as a result of (HER) signaling pathway inhibition or activation can be detected in vivo in human tumor bearing mice using anti-PD-L1 PET. Currently, multiple anti-PD-L1 tracers are investigated preclinically and clinically. If proven successful, anti-PD-L1 PET can, for example, be used to study which drugs most effectively decrease PD-L1 expression. Monitoring such effects might support decision making for combination treatments.

CONCLUSION
HER-targeted therapies and immunotherapy are both playing an important role in the treatment of cancer patients. Due to their complementary modes of action, HER-targeted therapies might be effectively combined with immunotherapy to improve clinical outcomes. This thesis demonstrates that studying the influence of HER-targeted therapies on the dynamics of HER proteins and immune checkpoints is of interest for the development of new cancer treatment strategies.

REFERENCES
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