Preclinical evaluation and molecular imaging of HER family dynamics to guide cancer therapy
Kol, Klaas Jan-Derk
CHAPTER 1
General Introduction
BACKGROUND
Cancer remains one of the leading causes of death worldwide (1). Current cancer treatment strategies consist of surgery, radiotherapy and systemic treatment. Using conventional multimodality therapy, the survival rates for certain tumor types still could benefit from major improvements (2). Moreover, the cancer incidence is increasing globally. This demonstrates an urgent need for new, effective anti-cancer therapies. During the past two decades, the paradigm for cancer treatment has expanded alongside relatively nonspecific chemotherapeutic drugs, such as taxanes and platinum-based anticaner drugs, to molecularly targeted therapeutics and cancer immunotherapy. 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. Targeted therapies can also have an effect on the immune system by affecting pathways that are crucial for immune development and function. Moreover, therapeutic monoclonal antibodies targeting specific tumor antigens can induce antibody-dependent cellular cytotoxicity (ADCC). Combining these approaches are part of current treatment strategies for many types of cancer, including lung, breast, colon, gastric, head and neck and ovarian cancer as well as melanoma.

One of the first known molecular targets has been the extensively studied human epidermal growth factor receptor (HER) family of receptor tyrosine kinases. The family comprising the epidermal growth factor receptor (EGFR) (also known as HER1), HER2, HER3 and HER4, is not only essential for the development and maintenance of normal tissues, but is also strongly involved in the development of many types of cancer (3,4). Upon ligand binding, these receptors homodimerize or interact with each other, forming heterodimers. Dimerization of the receptors results in activation of downstream signaling cascades such as the RAS-extracellular signal-regulated kinase (ERK) pathway and phosphoinositide 3-kinase (PI3K)/Akt pathway, thereby controlling many biological processes in normal tissue. However, these HER signaling pathways are frequently hyperactivated in cancer due to receptor overexpression, autocrine stimulation, crosstalk with other receptors and/or mutations in components of these pathways (5).

EGFR and HER2 are widely used targets for cancer therapy. In view of its overexpression in tumors and compensatory role in HER signaling, HER3 has gained much interest as an additional potential drug target as well. Drugs that block HER family members or inhibit downstream signaling by HER family members, including human anti-HER antibodies and tyrosine kinase inhibitors (TKIs), are increasingly becoming available. Anti-HER monoclonal antibodies can exert their action via a variety of mechanisms, including blocking (hetero)dimerization and ligand binding, as well as inducing HER endocytosis, complement dependent cytotoxicity (CDC) and ADCC (6–10). Several EGFR- and HER2-
targeted monoclonal antibodies and TKIs have already been approved by the Food and Drug Administration USA (11). Furthermore, a number of monoclonal antibodies that target HER3 have entered clinical trials (12). Nonetheless, intrinsic and acquired resistance against HER-targeted agents is a clinical problem, and much research focuses on optimizing targeting of the HER family (13,14).

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. HER-targeted agents affect many processes involved in HER protein expression, dynamics and availability, such as a) ligand binding, b) receptor dimerization, shedding, internalization, recycling to the cell surface and degradation, c) mobilization of internal receptor pools, and d) induction of HER family gene expression. In addition, the size of receptor-antibody complexes influences the amount of internalization and recycling, and subsequent degradation of receptors (15). A good example of these drug-induced changes in HER protein dynamics is the treatment of HER2-driven breast cancer with the EGFR/HER2 TKI lapatinib that has been shown to induce a transcriptional and posttranslational compensatory increase in HER3, which may attenuate antitumor efficacy of this drug (16).

Because of the dynamic HER membrane expression, a non-invasive dynamic assessment of HER tumor status in vivo will provide important supplementary information that cannot be obtained with invasive static techniques such as immunohistochemistry on biopsy material. Non-invasive molecular imaging, defined as the in vivo characterization and measurement of biological processes at the cellular and molecular level, could potentially be used as a tool to monitor receptor dynamics (17). 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 ($^{89}$Zr, t½ = 78.4 h), for optimal lesion accumulation. 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. Several (pre-) clinical studies have assessed in vivo treatment effects of drugs targeting HER2 such as lapatinib, trastuzumab or HSP90 inhibitors by quantifying the (change in) radiolabeled tracer accumulation in tumors (18–22). Results showed that these drugs reduced tumor uptake of radiolabeled anti-HER2 antibodies.

HER-targeted therapies can also influence dynamics of other plasma membrane proteins, such as immune checkpoints. Immune checkpoints refer to multiple co-stimulatory or inhibitory protein interactions that (among others) regulate T cell responses to antigens (23,24). These protein interactions are essential in self-tolerance and limit collateral
tissue damage when the immune system is responding to pathogenic infections. Tumors can exploit these immune checkpoints by upregulating inhibitory proteins and downregulating stimulatory proteins. This dampens T cell effector functions by inhibiting signaling downstream of the T cell receptor and, as a consequence, protects tumors from immune-mediated rejection. Programmed cell death 1 (PD-1) and its ligand PD-L1 have emerged as critical immune checkpoint proteins in cancer. PD-L1 expressed on tumor cells causes anergy and apoptosis of activated T cells by binding to PD-1 expressed on the surface of these T cells. PD-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, including heavily pretreated non-small cell lung cancer (NSCLC) and melanoma patients (25–27). Recently, it has been shown that EGFR plays a role in PD-L1 expression by tumor cells. EGFR inhibition reduces PD-L1 expression in vitro, resulting in a concomitant increase in T cell activation (28,29). Therefore, EGFR inhibition may be used to improve efficacy of immunotherapy.

Taken together, these results indicate that plasma membrane protein dynamics might well be important for proper design of (combination) therapies. Therefore, the aim of this thesis is to gain more insight in the effect of HER-targeting agents on HER and PD-L1 dynamics to provide a rationale for future combination therapies. In addition, HER tracers for molecular imaging to evaluate drug tumor targeting, organ distribution and target dynamics are explored.

OUTLINE OF THESIS
Anti-EGFR monoclonal antibody combinations can effectively inhibit the EGFR signaling pathway, and have shown superior anticancer efficacy in several human tumor xenograft models (30–32). Cetuximab and imgatuzumab are both monoclonal antibodies directed against distinct, non-overlapping epitopes in EGFR extracellular domain III. Imgatuzumab, in addition, is glycoengineered to enhance ADCC responses (33). Thus, combining these antibodies is a potential strategy to target EGFR more effectively than existing treatments with single antibodies. It is unknown whether treatment with imgatuzumab or the combination with cetuximab increases EGFR internalization and/or receptor membrane recycling in cancer cells, potentially reducing ADCC responses. In chapter 2, we investigate the effects of imgatuzumab and cetuximab on EGFR dynamics and intracellular signaling. In addition, we monitor whether changes in EGFR dynamics affect ADCC responses and tumor cell growth inhibition. Effects of imgatuzumab, cetuximab and the combination of these monoclonal antibodies on EGFR are studied in a panel of NSCLC cell lines. The effect of antibody binding on EGFR cell surface dynamics is determined using flow cytometry and immunofluorescence. In addition, the effect of antibody binding on total EGFR protein levels, downstream signaling and growth are
studied using Western blotting and proliferation assays. Finally, ADCC assays are used to investigate effects of EGFR dynamics on ADCC responses.

Whole body analysis of EGFR expression in tumor lesions using PET imaging could support decision making during clinical development and clinical practice. Preclinical EGFR-microPET imaging revealed a mismatch between \textit{in vivo} tumor EGFR expression assessed using Western blotting and tumor uptake of the radiolabeled EGFR antibody tracer (34). Shed EGFR ectodomain, which is present in cancer patient sera, can potentially bind tracer and therefore affect tracer kinetics (35). In \textbf{chapter 3}, we examine the influence of shed EGFR levels 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 are xenografted in mice. The A431 cell line is used, because of its known EGFR shedding potential. Tumor bearing mice receive various doses of $^{89}$Zr-imgatuzumab, co-injected with equal doses of $^{111}$In-IgG as internal control. MicroPET scans are performed at multiple time points post tracer injection, followed by biodistribution analysis. Shed EGFR levels in liver and plasma samples, as well as \textit{in vitro} culture media are determined by ELISA.

HER3 is the only member of the HER family having impaired tyrosine kinase activity and therefore its role in cancer has long been underestimated. However, in view of its overexpression in various tumor types and its compensatory role in HER signaling, HER3 has gained much interest as a potential target in cancer treatment. A review, \textbf{chapter 4}, describes the biology and relevance of HER3 in cancer, as well as drugs that block HER3 or interfere with HER3 heterodimer 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 PI3K pathway, which may attenuate the antitumor action of these inhibitors (13, 16, 36, 37). Preclinically, combining HER3 with HER2 inhibitors augmented tumor growth inhibition, \textit{in vivo} (38). This might advocate for a more dynamic assessment of HER3 tumor status than immunohistochemistry. Therefore, in \textbf{chapter 5}, we explore \textit{in vivo} HER3 tumor
status assessment with $^{89}$Zr-labeled anti-HER3 antibody mAb3481 PET after treatment with the dual EGFR/HER2 inhibitor lapatinib. The effect of lapatinib on HER3 cell surface expression and mAb3481 internalization is evaluated in human breast (BT474, SKBR3) and gastric (N87) cancer cell lines using flow cytometry. Next, mice bearing BT474 or N87 xenografts receive lapatinib daily for 9 days. PET-scans of xenografts with $^{89}$Zr-mAb3481 for visualization of HER3 are performed after 9 days lapatinib treatment. PET imaging and ex vivo organ distribution data are compared with Western blot and immunohistochemical stainings of tumor tissue.

Recently, it was shown that constitutively activated mutant EGFR induces PD-L1 expression in NSCLC cell lines (29). 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 (39). In contrast, PD-1 blockade has improved survival of EGFR wild-type NSCLC patients (39–41). Surprisingly, there is only limited data about the regulation of PD-L1 expression in EGFR wild-type NSCLC (42). Improved understanding of PD-L1 regulation may provide a rationale to combine immune checkpoint inhibitors with other targeted agents. Therefore, in chapter 6, we aim to identify pathways that regulate PD-L1 expression in EGFR wild-type NSCLC by using RNA-sequencing data from The Cancer Genome Atlas (TCGA) lung adenocarcinoma and squamous cell lung carcinoma data sets. We functionally validate our findings using a panel of EGFR wild-type lung adenocarcinoma cell lines and cocultures with peripheral blood mononuclear cells. We assess the effect of the combination interferon gamma (IFNγ), the most potent inducer of PD-L1 expression (43), and EGF, an activator of EGFR-mediated signaling, on PD-L1 mRNA, protein, and cell surface expression. Next, we investigate the effect of direct targeting of EGFR with cetuximab and erlotinib, or targeting of downstream signaling with XL147 (PI3K), everolimus (mammalian target of rapamycin 1 (mTORC1) and selumetinib (MAP/ERK kinase 1/2 (MEK1/2)) on PD-L1 expression. Effects on PD-L1 expression and downstream signaling are studied using qPCR, Western blotting and flow cytometry.

In chapter 7, the findings of this thesis are summarized, followed by a general discussion with future perspectives.

REFERENCES
2. Mathers CD, Loncar D. Projections of global mortality and burden of disease from


