Preclinical evaluation and molecular imaging of HER family dynamics to guide cancer therapy
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CHAPTER 4

HER3, serious partner in crime: Therapeutic approaches and potential biomarkers for effect of HER3-targeting

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ABSTRACT
The human epidermal growth factor receptor (HER) family members are targeted by a growing numbers of small molecules and monoclonal antibodies. Resistance against the epidermal growth factor receptor (EGFR) and HER2-targeting agents is a clinically relevant problem forcing research on optimizing targeting of the HER family. In view of its overexpression in tumors, and compensatory role in HER signaling, HER3 has gained much interest as a potential additional target within the HER family. It is the only member of the HER family lacking intrinsic tyrosine kinase activity and therefore its role in cancer has long been underestimated. Drugs that block HER3 or interfere with HER3 dimer signaling, including fully human anti-HER3 antibodies, bispecific antibodies and tyrosine kinase inhibitors (TKIs), are currently becoming available. Several compounds have already entered clinical trial. In the meantime potential biomarkers are tested such as tumor analysis of HER3 expression, functional assays for downstream effector molecules and molecular imaging techniques. This review describes the biology and relevance of HER3 in cancer, agents targeting HER3 and potential biomarkers for effect of HER3-targeting.
INTRODUCTION
The human epidermal growth factor receptor (HER) family comprising the epidermal growth factor receptor (EGFR) (also known as HER1), HER2, HER3 and HER4 (also known as respectively ErbB2, ErbB3, and ErbB4) is not only essential for development and maintenance of normal tissue, but is also strongly involved in the development of many tumor types (Campbell et al., 2010). EGFR and HER2 are widely known targets for cancer therapy, with both monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs) directed against these receptors. The mAb trastuzumab which targets HER2 is now part of standard of care for patients with HER2-positive breast cancers (Yeon and Pegram, 2005). However, intrinsic or developed resistance against HER-targeting agents, is a clinical problem, and much research focuses on optimizing targeting of the HER family (Garrett and Arteaga, 2011 a; Sergina et al., 2007).

HER3 is the only member of the HER family lacking intrinsic tyrosine kinase activity. Therefore its role in cancer has long been underestimated. EGFR and HER2 are the preferred dimerization partners of HER3 (Prenzel et al., 2001; Graus-Porta et al., 1997). Interestingly, these two HER3 heterodimers are the most active signaling complexes in this family (Tzahar et al., 1996; Holbro et al., 2003). As such, HER3 is implicated in tumor growth and maintenance of many solid tumor types. In view of its overexpression in tumors, and compensatory role in HER signaling, HER3 is an interesting target to optimize HER family signaling inhibition. Anti-HER3-targeting agents, such as anti-HER3 mAbs and bispecific antibodies targeting both HER3 and EGFR, are currently in development. HER3 mAbs can inhibit ligand-induced phosphorylation of HER2, HER3 and downstream effector molecules, including the extracellular-signal-regulated kinase (ERK) 1, ERK2 and AKT. Blocking of HER3 dimer dependent signaling can furthermore be achieved with TKIs targeting EGFR and HER2, which indirectly inhibit HER3 phosphorylation. In the meantime potential biomarkers are tested, including tumor analysis of HER3 expression, functional assays for downstream effector molecules and molecular imaging techniques.

Therefore, the focus of this review is to describe the biology and relevance of HER3 in cancer, the currently known agents targeting HER3 and potential biomarkers for effect of HER3-targeting.

HER3, A MEMBER OF THE HER FAMILY
Receptor characteristics
HER3 is encoded by the ERBB3 gene and maps to the human chromosome 12q13. Its mRNA gives rise to a 185 kDa transmembrane glycoprotein, which is composed of three regions: a NH2-terminal extracellular ligand-binding region, a transmembrane domain, and an intracellular region containing the COOH-terminal (Zimonjic et al.,
HER3 has marginal kinase activity due to substitutions in its tyrosine kinase domain, and efficient downstream signaling reportedly happens as a result of heterodimerization (Shi et al., 2010; Sergina et al., 2007; Plowman et al., 1990). Furthermore, it is not transforming when constitutively activated by continuous ligand stimulation or when it is overexpressed (Zhang et al., 1996). Recently, ERBB3 somatic mutations have been found in several types of human cancer, including colon and gastric cancer (Jaiswal et al., 2013).

HER3 is physiologically expressed in a wide variety of normal human tissue, including cells of the gastrointestinal, urinary, respiratory, and reproductive tracts as well as the skin, endocrine and nervous system (Prigent et al., 1992). In the pathological setting, overexpression of HER3 is often accompanied by overexpression of EGFR and/or HER2 (Ito et al., 2001; Giltnane et al., 2009). Furthermore, HER3 is a co-receptor for the amplified HER2 in breast cancer (Holbro et al., 2003) and is strongly implicated as a co-receptor for EGFR in a subset of EGFR-driven lung cancers and seems to be an effective predictor of sensitivity to the EGFR TKI gefitinib (Engelman et al., 2005). Immunohistochemical studies showed that HER3 overexpression is often associated with poor prognosis in various tumor types including breast, head and neck and gastric cancer (Giltnane et al., 2009; Takikita et al., 2011; Hayashi et al., 2008).

Eleven ligands bind to the HER family members, including epidermal growth factor (EGF), transforming growth factor α (TGF-α) and heregulin (HRG)/neuregulin (NRG) family members (Harris et al., 2003). HER2 has no identified ligands and exists in an open conformation that allows dimerization with other HER members. The primary ligands for HER3 are the members of the NRG family, including NRG1 (also known as HRG). Ligands can bind directly to the extracellular domain of EGFR, HER3 or HER4, which leads to a conformational rearrangement. This rearrangement exposes the dimerization domain that forms the core of the dimer interface with another HER. In HER2 amplified cancers, HER2:HER3 dimers may also be formed in a ligand-independent manner (Mukherjee et al., 2011). After the heterodimerization with other HER family members, the tyrosine kinase portion of HER3 becomes transphosphorylated. The phosphorylation creates docking sites that allow the recruitment of downstream signaling proteins. These signaling proteins include SH2 containing protein (SHC) and growth factor receptor-bound protein 7 (GRB7) that activate the RAS-mitogen activated protein kinase RAS-MAPK pathway (see Fig. 1). Apart from activating the RAS-MAPK pathway, the HER3 cytoplasmic domain contains 6 docking sites for phosphatidylinositol 3-kinase (PI3K) (Campbell et al., 2010). When PI3K is activated by a HER3 dimer, it phosphorylates membrane phosphatidylinositol bisphosphate (PIP2), which forms phosphatidylinositol trisphosphate (PIP3). This leads to the recruitment and subsequently to the activation
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of phosphoinositide-dependent kinase-1 (PDK1) and AKT. AKT is able to activate the mammalian target of rapamycin (mTOR) - a downstream mediator of the PI3K/AKT pathway - and thereby controls many biological processes, which are important for tumorigenesis. These biological processes include survival, translation, nutrient sensing, cell cycle control and metabolic regulation.

HER3 also plays a compensatory role in HER signaling. HER3 expression or signaling is associated with resistance to HER2 inhibitors in breast cancers. Treatment of HER2-driven breast cancers cell lines and xenograft tumors with HER-targeting TKIs led to a rapid compensatory increase in expression, signaling activity and relocalization of HER3.
to the plasma membrane (Sergina et al., 2007). The compensatory increase in signaling activity is due to a compensatory shift in the HER3 phosphorylation–dephosphorylation equilibrium. This is caused by increased membrane HER3 expression (driving the phosphorylation reaction) and by reduced HER3 phosphatase activity (decreasing the dephosphorylation reaction). The knockdown of HER3 using small interfering RNA (siRNA) restored the potent pro-apoptotic activity of the TKIs. In addition, inhibition of HER3 with siRNA or a neutralizing HER3-antibody sensitized HER2-positive breast cancer cells and xenografts to lapatinib, which blocks the activation of both HER2 and EGFR, in vitro and in vivo. Therefore, persistent and complete inhibition of HER3 and its output to PI3K/AKT are likely needed for the optimal antitumor effect of therapeutic inhibitors of HER2 (Garrett et al., 2011 b; Garrett et al., 2013a).

The kinase domain of HER3 has long been assumed to be inactive and has been classified as a pseudokinase (Citri, 2003; Boudeau et al., 2005). However, there is evidence that it is able to bind ATP and promote trans-autophosphorylation of the receptor’s intracellular domain when it is clustered at a membrane surface (Shi et al., 2010). The tyrosine kinase activity is roughly 1,000-fold weaker than that of EGFR. This might be sufficient for receptor transphosphorylation in the context of a heterodimer with another family member. In vitro, HER3 autophosphorylation was not inhibited by the TKIs lapatinib, gefitinib or erlotinib. In addition, HER3, but not HER2, is able to phosphorylate proline-rich tyrosine kinase 2 (PYK2) due to a very selective substrate specificity (van der Horst et al., 2005). PYK2 is a cytoplasmic tyrosine kinase, which is highly expressed in the central nervous system and promotes migration and invasion of glioma cells (Lipinski et al., 2005). The phosphorylation of PYK2 leads to a mitogenic response through activation of the MAPK pathway in human glioma cells (van der Horst et al., 2005). Expression of a dominant-negative PYK2 construct abrogated the HRG-induced MAPK activity, leading to the inhibition of the invasive potential of glioma cells. Activation of HER3, without detection of other activated HER family members, was reported in other cancer cell lines such as the breast cancer cell lines BT483, T47D and MCF-10A, and the ovarian cancer cell line OVCAR (Rajkumar and Gullick, 1994; Beerli et al., 1995). Other kinases such as the MET receptor may also activate HER3 under some circumstances. In vitro, MET activated HER3 signaling in a gefitinib resistant cell line (Engelman et al., 2007).

HER3 is usually localized at the cell membrane as a transmembrane receptor tyrosine kinase (RTK) and is activated by extracellular binding of its ligands. However, membranous HER3 is able to move to the nucleus and this event may play an important role in the progression of cancer. HER3 was detected in the nuclei of nonmalignant and human breast cancer cells (Offterdinger et al., 2002). Additionally, the nuclear export inhibitor leptomycin B caused accumulation of HER3 in the nuclei. Immunohistochemistry and
Western blotting showed that HER3 was also expressed in the nuclei of prostate cancer cell lines and prostate tumor tissue (Koumakpayi et al., 2006). In contrast, there was minimal HER3 nuclear staining in normal prostate and benign prostatic hyperplasia tissues. This nuclear expression correlated positively with the Gleason grade of prostate cancers. The nuclear HER3 localization was higher in hormone refractory compared to hormone-sensitive human prostate cancers. However, nuclear localization of HER3 does not necessarily lead to unfavorable tumor characteristics. Paraffin-embedded samples from 128 consecutive uveal melanoma patients, enucleated without alternative treatment at diagnosis, were evaluated for HER3 using immunohistochemistry. Solely nuclear localization of HER3 was present in 56 of the 89 HER3 positive tumors and predicted a more favorable overall survival (Trocmé et al., 2012). Translocation of HER3 to the nucleus may be an important step in its signaling process. Depleting tumor cells in vitro of ATP or glucose or treating with chemotherapy such as cisplatin, resulted in increased nuclear HER3 expression (Schormann, 2008).

EGFR and HER2 can also be localized in the nucleus. Here they play a role in transcriptional regulation, cell proliferation, metastasis, DNA repair and chemoresistance and radio-resistance (Lin et al., 2001; Hanada et al., 2006; Lo et al., 2005; Wang et al., 2004). Because HER members have similar functional domains, HER3 could also have a potential role as a transcription factor involved in cell proliferation and tumor progression. Indeed, HER380 kDa, a nuclear variant of the HER3, binds to the cyclin D1 promoter and therefore activated cell proliferation in H358 lung cancer cells (Andrique et al., 2012). This nuclear translocation occurred independently from HRG binding and was negatively controlled by the tumor suppressor functions of p14ARF.

TKIs and mAbs targeting the ligand binding site, the ATP binding site or the dimerization domain might be unable to interfere with the nuclear pathways controlled by the nuclear HER variants. The underlying mechanisms for nuclear localization remain unknown. As subcellular localization was not taken into account in many studies, it might be possible that discrepancies related to HER3 expression analysis in cancer tissues could partially be caused by this factor.

Therefore in conclusion, HER3 is characterized by its potent ability to activate downstream PI3K and AKT pathway signaling, and is implicated in the development and progression of cancer. Although attempts at targeting HER3 in cancers have lagged behind, partially due to its marginal kinase activity, the important role for HER3 as a signaling hub for the other HER family members in cancer is becoming increasingly apparent. Recent evidence that inhibiting the HER signaling network can lead to a rapid compensatory increase in HER3 expression highlights the relevance of HER3 as a therapeutic target.
Relevance of HER3 in cancer

The relevance of HER3 in cancer has been studied in several tumor types, including breast, lung, gastric, head and neck, ovarian cancer and melanoma. HER3 overexpression was found in 10% of the tumors of more than 4,000 patients diagnosed with invasive breast cancer. With a median follow-up of 12.5 years, HER3 overexpression was related to reduced survival ($P < 0.0001$) (Chiu et al., 2010). The role of HER3 as partner in crime is emphasized in vitro by its rapid increase in breast cancers cells and xenograft tumors after treatment with HER2-targeting TKIs, and the need to completely inhibit HER3 for optimal anti-HER2 targeting (Garrett et al., 2011 b; Garrett et al., 2013a). A cohort study in the particularly aggressive breast cancer subtype inflammatory breast cancer included patients with stage III and stage IV disease. A favorable response to the TKI lapatinib was related to co-expression of phosphorylated (p) HER2 and pHER3. pHER2 was present in 11 out of 12 responders and 13 out of 14 non-responders while pHER3 was present in 10 out of 12 responders versus 5 out of 14 non-responders ($P = 0.021$). Presumably, this co-expression reflects the activated status and signaling-competent state of HER2 in inflammatory breast cancer, which could explain the predictive value of pHER3 for lapatinib sensitivity in this setting (Johnston et al., 2008). HER3 plays also a potential role in endocrine resistance in breast cancer. The estrogen receptor (ER) pathway plays an essential role in breast cancer development and progression. Endocrine therapies, such as the antiestrogens fulvestrant and tamoxifen, which block the ER pathway, are highly effective, but their activity in the metastatic setting is limited by intrinsic and eventually acquired resistance in all patients. Exposure of ER-positive breast cancer cell lines MCF-7 and T47D to fulvestrant induced the expression of HER3 and sensitized them to HRG (Hutcheson et al., 2011). A study retrospectively analyzed tamoxifen treated (N = 352) ER-positive breast cancer patients and showed that those with HER3-overexpression (N = 56) were more likely to have shorter progression-free survival ($P = 0.0278$) (Tovey et al., 2005).

In primary non-small cell lung cancers (NSCLC) (adenocarcinoma, squamous cell carcinoma and large cell carcinoma), 443 tumors were immunostained for HER3 expression. High HER3 expression correlated with a shorter overall survival in patients with stage III and IV ($P = 0.002$), but not in stage I or II disease (Yi et al., 1997).

The HER pathway is also of clinical importance in gastric cancer. Combination therapy consisting of trastuzumab with chemotherapy showed superiority in patients with HER2 overexpressing advanced gastric carcinoma compared to chemotherapy alone (Cervantes et al., 2013). Immunohistochemistry and fluorescence in situ hybridization of HER3 were performed in 191 patients, who underwent surgery between 1998-2006.
for gastric cancer and showed that HER3 expression was related to worse overall survival ($P = 0.035$) (Begnami et al., 2011). Here, HER3 expressed in the nucleus predicted poor survival. Numeric alterations in the HER3 gene or chromosome 12 by FISH were not found.

Positive membranous HER3 tumor expression was detected in 34 of 378 (8.8%) head and neck squamous cell carcinoma (HNSCC) patients and was associated with worse overall survival ($P = 0.027$). Interestingly, membranous HER3 overexpression was increased in metastases compared to primary tumors ($P = 0.003$) (Takikita et al., 2011). Regrettably, this study lacked staging information of the patients. Recently, it was shown that HNSCC tumors express the highest levels of HRG in tumors of diverse origin, including NSCLC, melanoma and ovarian cancer. High HRG expression was associated with activated HER3. Additionally, higher distribution of HRG was expressed by recurrent HNSCC tumors versus primary tumors, or in post-therapy recurrence versus therapy naïve tumors (Shames et al., 2013).

In melanoma, HER3 is frequently overexpressed (Reschke et al., 2008). Moderate to high HER3 expression levels were found in 85 of 130 (65%) primary tumors and HER3 was highly expressed in 35 of 87 (40%) metastases. A multivariate analysis showed that HER3 expression correlated with poor prognosis ($P = 0.041$). To assess the role of HER3 in migration and invasion, melanoma cells were seeded either on a membrane or on a growth factor–reduced Matrigel coated membrane with 8 µm pores. HRG-β1 was used as a chemoattractant. The suppression of HER3 expression by siRNA, as well as mAbs against HER3, reduced melanoma cell proliferation, migration and invasion (Reschke et al., 2008).

High HER3 expression in 62 out of 116 patients with primary epithelial ovarian cancer (FIGO stages I-IV), who were treated primarily by surgery, correlated with decreased survival ($P = 0.0034$) (Tanner et al., 2006). However, there was no association between HER3 expression and FIGO stage. In addition, ovarian cancer patients with high HER3 mRNA levels, who were treated with gemcitabine and the HER2 dimerization inhibitor pertuzumab, showed a trend towards earlier progression of disease compared to those with relatively low levels of HER3 mRNA (Makhija et al., 2010). This might indicate that HER3 also plays a role in ovarian cancer. There is a remarkable discrepancy in percentage positive staining between ovarian cancer studies. Positive HER3 staining was observed in 53.4% of the patients with primary epithelial ovarian cancer (FIGO stages I-IV) (Tanner et al., 2006), whereas only 3% were positive in patients with FIGO stage III or IV who were treated with platinum-taxane chemotherapy (Lee et al., 2005). In another study, 62 of 73 samples (85%), showed positive HER3 staining in ovarian tumors (Simpson et
This discrepancy might be caused by the use of different HER3 antibodies and antigen retrieval methods. There are currently no standardization guidelines for these studies using HER3 antibodies. In 42 lung tumors, HER3 was measured by six different antibodies. In this study, HER3 staining was nonspecific and nonreproducible (Anagnostou et al., 2010). This highlights the importance of IHC standardization.

**TARGETING HER3 SIGNALING**

It is unclear whether ATP binding is required or plays a role in the stimulatory function or stability of the HER3 kinase domain. Therefore, the ATP analogue class of TKIs may not bind to this target. However, other strategies are potentially valid, such as targeting the receptor with HER3 antibodies, blockade of (signaling of) HER3 dimers with other HER family members, and targeting HER3 ligands. In this paragraph, we will describe the evidence available at present, for these three options. An overview of HER3 targeting therapies and their site(s) of action is shown in Fig. 2.

**Anti-HER3 antibodies**

Currently, there are several HER3 targeted mAbs in development, which exert their action via a variety of mechanisms. These include blocking the heterodimerization with the HER family members EGFR, HER2 and HER4, blocking the binding of the HER3 ligand HRG to the receptor and downregulation of membranous HER3. Furthermore, these mAbs are able to engage immune effector functions, which can lead to antibody dependent cellular cytotoxicity (ADCC). Most of these anti-HER3 antibodies are of the human IgG1 or IgG2 isotype. ADCC is usually considered an important mechanism of action for immunotherapy with human IgG1 but not IgG2 antibodies. However, IgG2 antibodies can trigger ADCC but, in contrast to IgG1, only by cells of myeloid lineage such as neutrophils and monocytes (Schneider-Merck et al., 2011). Most of the anti-HER3 antibodies recognize only HER3. A subset of them has been engineered to bind not only HER3 but also an additional RTK. Furthermore, HER3 can be inhibited by pertuzumab, which binds to HER2 and thereby prevents the dimerization with HER3. This section will only describe the currently studied anti-HER3 antibodies in clinical trials and are shown in Table I. Pertuzumab does not bind to HER3, and therefore will be discussed in the section on Blockade of HER3 signaling.

The fully humanized HER3 antibody U3–1287 (AMG 888) demonstrated in vitro and in vivo efficacy of reduced growth in the xenografted human HNSCC FaDu model. In vitro, AMG 888 prevented ligand-induced phosphorylation of HER3, HER2, and downstream effector molecules including AKT, ERK1 and ERK2. A combination with anti-EGFR mAb panitumumab showed synergy in the xenografted FaDu model (Freeman et al., 2011). AMG 888 is currently in phase II development and is in clinical trial in combination with
Figure 2:
Inhibition of HER3 signaling. Various compounds can block HER3 directly or indirectly interfere with HER3 dimer signaling. These compounds include antibodies, such as MM-111, pertuzumab and MEHD7945A, that inhibit HER2/HER3 and EGFR:HER3 dimer signaling. But also antibodies that only target the HER3 receptor such as AMG 888, MM-121, AV-203, LJM716 and RO547959. RO547959 differs from the other anti-HER3 antibodies because of its glycosylated Fc region. This results in a higher affinity to the human FcγRIIIa on immune effector cells.

MM-141 is a bispecific antibody which co-targets HER3 and IGF-1R. TKIs such as erlotinib and gefitinib, which inhibit EGFR, lapatinib and AZD8931, which inhibit EGFR and HER2, and afatinib, which inhibits EGFR, HER2 and HER4 (might) indirectly inhibit HER3 signaling. The HDACi entinostat can reduce the levels of HER2, HER3, pHER2, pHER3, p-AKT and p-MAPK. Finally, the HSP90 inhibitor 17-AAG inhibits conformational maturation and refolding of a variety of signaling proteins including the HER family, as well as downstream signaling components and thereby inhibits HER3 signaling.
trastuzumab and paclitaxel in newly diagnosed HER2-positive metastatic breast cancer (NCT01512199) and with or without erlotinib in advanced NSCLC (NCT01211483).

The human monoclonal HER3 antibody MM-121 inhibited proliferation of pancreatic ductal adenocarcinoma in vitro (Liles et al., 2011). A combination of MM-121 with the TKI erlotinib completely abolished AKT activation in pancreatic cancer cells. MM-121 reduced basal HER3 phosphorylation most effectively in cancer cell lines that possess ligand-dependent activation of HER3, but not in cell lines with HER2 amplification (Schoeberl et al., 2010). In vivo, MM-121 inhibited proliferation in the xenografted pancreatic cancer AsPC-1 model and ovarian cancer OVCAR8 model (Liles et al., 2011; Sheng et al., 2010). Resistance to platinum-based drugs can arise through upregulation of HER3 signaling and activation of the PI3K/AKT pathway. In addition, platinum resistance is associated with increased HRG expression or responsiveness to HRG in the cisplatin resistant human ovarian cancer cell line A2780-cis and primary ascites cells isolated from advanced stage ovarian cancer patients who had undergone prior therapy. The growth of A2780-cis (but not A2780) xenografts was inhibited by MM-121. In most cases, ascites cells that were responsive to HRG stimulation could also be inhibited by MM-121. MM-121 sensitized some of these cells to paclitaxel treatment (Curley et al., 2012). In addition, activation of HER3 is associated with resistance to anticancer agents such as PI3K-AKT pathway kinase inhibitors in human breast cancer cell lines (Chakrabarty et al., 2011) and tubulin binders such as paclitaxel in HER2 overexpressing breast cancer cells (Wang et al., 2010). Therefore, the anti-tumor effect of a pan-PI3K inhibitor or a microtubule inhibitor in combination with MM-121 was tested in mice. MM-121 showed synergistic tumor growth inhibition in combination with the pan-PI3K inhibitor SAR245408 in mice bearing human lung carcinoma A549 tumors and in combination with the microtubule inhibitor cabazitaxel in mice bearing human gastric N87 HER2-overexpressing tumors (Henry et al., 2012). MM-121 is currently in a phase I clinical trial in solid tumor patients in combination with the pan-PI3K inhibitor SAR245408 (NCT01436565) or cabazitaxel (NCT01447225).

Table 1
HER3 targeting drugs in clinical trials.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Sort of drug</th>
<th>Stage of clinical development</th>
<th>Pharmaceutical company</th>
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<tbody>
<tr>
<td>AMG 888 (U3-1287)</td>
<td>Fully human monoclonal antibody</td>
<td>Phase II</td>
<td>Amgen/Daiichi Sankyo</td>
</tr>
<tr>
<td>MM-121</td>
<td>Fully human monoclonal antibody</td>
<td>Phase I</td>
<td>Merrimack Pharmaceuticals/Glaxo</td>
</tr>
<tr>
<td>RGS69559 (GE-huMab-HER3)</td>
<td>Glycoengineered humanized monoclonal antibody</td>
<td>Phase I</td>
<td>Boehringer Ingelheim Pharmaceuticals</td>
</tr>
<tr>
<td>MM-111 (Anti-HER2/HER3)</td>
<td>Bispecific antibody</td>
<td>Phase I</td>
<td>Merrimack Pharmaceuticals</td>
</tr>
<tr>
<td>MDR7945A (Anti-HER3/EGFR)</td>
<td>Bispecific antibody</td>
<td>Phase II</td>
<td>Genentech</td>
</tr>
<tr>
<td>AV-203 (Humanized monoclonal antibody)</td>
<td>Humanized monoclonal antibody</td>
<td>Phase I</td>
<td>AVEO Oncology</td>
</tr>
<tr>
<td>MM-141 (Anti-HER3/IGF1R)</td>
<td>Fully human bispecific antibody</td>
<td>Phase I</td>
<td>Merck</td>
</tr>
<tr>
<td>AF2360 (BIBW 2992)</td>
<td>TKI</td>
<td>FDA approved</td>
<td>Boehringer Ingelheim Pharmaceuticals</td>
</tr>
<tr>
<td>AZD9511 (BIBW 2992)</td>
<td>TKI</td>
<td>Phase II</td>
<td>AstraZeneca</td>
</tr>
<tr>
<td>Enzastaurin (SNDX-275)</td>
<td>HDACI</td>
<td>Phase II</td>
<td>Syndax Pharmaceuticals</td>
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</tbody>
</table>
AV-203 is a humanized monoclonal HER3 antibody which inhibits the binding of HRG to HER3, promotes HER3 degradation and inhibits tumor growth in human breast, lung, kidney and pancreas xenograft models (Meetze et al., 2012). This antibody is currently in a phase I clinical trial (NCT01603979).

The bispecific antibody MM-111 was developed to overcome the limited inhibition of ligand-induced HER3 activation and phosphorylation by agents such as lapatinib, trastuzumab and pertuzumab. This antibody targets HER3, by forming a trimeric complex with HER2 and HER3. In the BT-474-M3 HER2 overexpressing breast cancer xenograft model, MM-111 induced antiproliferative effects. The combination of MM-111 with trastuzumab or lapatinib more potently inhibited tumor growth in the xenograft model than the monotherapies (McDonagh et al., 2012). A phase I study of MM-111 in 30 patients with advanced HER2-positive solid tumors showed that MM-111 could be safely combined with standard of care HER2-targeting drugs such as trastuzumab and lapatinib (Richards et al., 2012). Currently, studies are ongoing in combination with trastuzumab in patients with advanced, HER2 amplified, HRG-positive breast cancer (NCT01097460) and in combination with multiple trastuzumab containing combination regimens in patients with HER2-positive cancer (NCT01304784).

The bispecific antibody against EGFR and HER3, called MEHD7945A, showed preclinical efficacy. In vitro, this compound inhibits the growth of human HNSCC and NSCLC cell lines that exhibit resistance to cetuximab and erlotinib (Shyhmin et al., 2011). Furthermore, in human tumor xenograft models, MEHD7945A showed tumor growth inhibition (Schaef er et al., 2011) and overcame resistance to cetuximab or erlotinib (Huang et al., 2013). In a phase I trial pharmacodynamics data indicated target inhibition in 8 out of 36 patients (HNSCC = 2, NSCLC = 2, colorectal cancer (CRC) = 4), and best response by RECIST included 2 partial response (both HNSCC) and 6 patients with stable disease of more than 8 weeks (NSCLC = 2, CRC = 4) (Cervantes-Ruiperez et al., 2012). MEHD7945A is currently in a phase II trial compared with cetuximab in patients with recurrent/metastatic HNSCC (NCT01577173). In addition, MEHD7945A will be evaluated in combination with FOLFIRI chemotherapy versus cetuximab in combination with FOLFIRI in patients with K-RAS wild-type metastatic colorectal cancer (NCT01652482).

The glycoengineered (GE) humanized monoclonal HER3 antibody (GE-huMab-HER3, RO5479599) differs from other anti-HER3 antibodies because of its glycosylated Fc region. This results in a 50-fold higher affinity to the human FcγRIIIa on immune effector cells compared to standard IgG1 antibodies. The additional engagement of immune effector functions may explain the improved efficacy of this compound compared to a non-glycoengineered variant in the A549 (human lung adenocarcinoma cell line) orthotopic mouse
models bearing murine monocytes, macrophages and/or NK cells as immune effector cells (Mirschberger et al., 2013). Its efficacy was further enhanced in combination with anti-EGFR or anti-HER2 monoclonal antibodies. GE-huMab-HER3 is currently in phase I development as single agent and in combination with cetuximab or erlotinib in patients with metastatic and/or locally advanced HER3-positive epithelial tumors (NCT01482377). Preliminary data revealed that GE-huMab-HER3 monotherapy was well tolerated and 5 out of 25 patients had stable disease as best response (Meulendijks et al., 2013).

The human HER3 antibody LJM716, which was selected from a human combinatorial antibody library for its ability to neutralize multiple modes of HER3 activation, binds to a novel conformational epitope and traps HER3 in the inactive conformation. This antibody does not block HRG binding to HER3 and does not affect the binding affinity of the HER3/HRG interaction. LJM716 is able to inhibit HER3/AKT phosphorylation and proliferation in a range of HER2 amplified and HRG expressing cell lines in vitro (Garner et al., 2013). In vivo, LJM716 induced tumor regression in FaDu xenografts and tumor growth inhibition (>80%) in xenograft models including BT474 (HER2 amplified breast cancer). Combination of LJM716 with cetuximab, trastuzumab or PI3K-targeted agents was synergistic in a panel of cell lines while the combination of LJM716 with trastuzumab or erlotinib was effective in L3.3 (pancreatic cancer) and BT474 xenograft models (Garner et al., 2012; Garrett et al., 2013b). LJM716 is currently in phase I development in patients with HNSCC, HER2-positive breast cancer or gastric cancer (NCT01598077).

Many cancer cell lines rely on insulin-like growth factor 1 (IGF-1) and HRG signaling in a redundant fashion to support their survival and proliferation. Blocking IGF-1R with mAbs in early clinical development gave disappointing results, leading some companies to discontinue clinical trials focusing on this receptor. HRG secretion or HER3 upregulation can compensate for the IGF-1/IGF-1R blockade. Therefore, a novel fully human bispecific antibody, MM-141, which co-targets HER3 and IGF-1R, has been developed. MM-141 can inhibit proliferative and metabolic signals in cancer cells. It furthermore blocks IGF-1, IGF-2, and HRG binding to IGF1-R and HER3. This leads to a downregulation of receptor homodimers as well as IGF-1R/insulin receptor, EGFR/HER3 and HER2/HER3 heterodimers. In vivo, MM-141 showed monotherapy activity in xenograft models of prostate cancer (DU145), sarcoma (SK-ES-1), pancreatic (BxPC-3) and renal cell cancer (Caki-1) and showed increased activity compared to monospecific antibodies and their combination in tumors with IGF-1R and HER3 signaling (Baum et al., 2012). MM-141 is currently evaluated in a phase I trial as single agent and in combination with everolimus and docetaxel (NCT01733004).
Blockade of HER3 signaling

HER3 phosphorylation requires dimerization with other HER family members. Targeting these dimers can be obtained by humanized mAbs, that bind to the extracellular domain of HER2 and thereby prevent the dimerization of HER2 with HER3 and other HER family receptors. Pertuzumab, a registered drug for the treatment of HER2-positive metastatic breast cancer, is such an antibody. Monotherapy using pertuzumab has limited clinical efficacy, but particularly the addition of pertuzumab to trastuzumab-based regimens improves outcome in patients with HER2-positive breast cancer. In the landmark NeoSphere trial, adding pertuzumab to trastuzumab and docetaxel improved pathological complete response rates (in 49 of 107 patients versus 31 of 107, \( P = 0.0141 \)) in locally advanced, inflammatory or early HER2-positive breast cancer (Gianni et al., 2012). This finding was confirmed in the CLEOPATRA trial in 808 HER2-positive metastatic breast cancer patients who were randomly assigned to receive placebo plus trastuzumab plus docetaxel or pertuzumab plus trastuzumab plus docetaxel. Addition of pertuzumab improved progression-free survival from 12.4 to 18.5 months (\( P < 0.001 \)) (Baselga et al., 2012 c). Pertuzumab in combination with trastuzumab also showed promising effects in preclinical models of gastric cancer, and is worth examining as a new therapy for HER2-positive gastric cancer (Yamashita-Kashima et al., 2011). In a phase II study with pertuzumab in combination with gemcitabine, ovarian cancer patients with low HER3 mRNA showed increased progression-free survival in the gemcitabine plus pertuzumab arm compared to gemcitabine alone (Makhija et al., 2010). The 26 patients with low HER3 mRNA levels who received gemcitabine in combination with pertuzumab had a progression-free survival of 5.3 months compared to 1.4 months in 35 patients receiving gemcitabine alone (\( P = 0.0002 \)).

Further downstream blocking of HER3 dimer dependent signaling can be achieved with TKIs. Erlotinib, a low molecular weight, orally bioavailable inhibitor of EGFR, blocks HER3 phosphorylation in pancreatic and CRC cell lines. This is presumed to be related to disruption of the EGFR/HER3 heterodimer (Buck et al., 2006). In HER2-positive breast cancer particularly the combination of trastuzumab and lapatinib has additive efficacy, both in the metastatic as well as in the neo-adjuvant setting (Blackwell et al., 2012; Baselga et al., 2012 a). In patients with HER2-positive metastatic breast cancer, progressing on trastuzumab based treatment (\( N = 291 \)), trastuzumab and lapatinib improved overall survival from 9.5 to 14 months (\( P < 0.26 \)), compared to lapatinib alone (Blackwell et al., 2012). This is remarkable, particularly in view of the heavy pretreatment in these patients, and even despite cross-over between treatment arms. In the neo-adjuvant setting, the pathological complete response was higher (\( P = 0.0001 \)) in the group receiving the combination therapy (78 of 152 patients) compared to those receiving trastuzumab (44 of 149 patients) or lapatinib alone (38 of 154 patients) (Baselga et al., 2012 a).
Afatinib (BIBW 2992) is a potent, small molecule HER family blocker that covalently binds and irreversibly blocks signaling through activated EGFR, HER2 and HER4, as well as the transphosphorylation of HER3 (Solca et al., 2012). In comparison to gefitinib and erlotinib, afatinib had superior activity in human lung cancer cells expressing EGFR-activating mutations, and in vivo increased antitumor activity in human lung cancer xenograft models (Li et al., 2008). In a phase II study with 41 HER2-positive, trastuzumab-resistant breast cancer patients, 4 patients had partial response and 15 stable disease (Lin et al., 2012). In another phase II study with 41 NSCLC patients, 1 confirmed partial response was observed and 24 patients had stable disease (De Greve et al., 2013). Afatinib is approved by the U. S. Food and Drug Administration for the first-line treatment of patients with metastatic NSCLC whose tumors have EGFR exon 21 (L858R) substitution mutations or exon 19 deletions. Currently, afatinib is in phase III development and evaluated as maintenance therapy in HNSCC (NCT01427478).

The TKI AZD8931 showed inhibition of p-EGFR, p-HER2, p-HER3, survival signaling pathways (p-ERK and p-AKT) and growth in a panel of human cancer cells in vitro and in vivo (Hickinson et al., 2010). AZD8931 also reduced cell growth in trastuzumab-resistant BT474 and SKBR3 cell lines (Balachandran et al., 2012). When AZD8931 was tested in combination with trastuzumab, the resistance to trastuzumab was reversed. A phase II study (NCT00900627) with AZD8931 did not meet its primary objective of prolonging progression-free survival when it was added to weekly paclitaxel in patients with low HER2-expressing advanced breast cancer (Baselga et al., 2013).

These studies have enforced the concept of dual targeting of more than one HER family member simultaneously to circumvent (presumably HER3 driven) resistance. Preclinical data, implying that pHER3 only decreases with the combination of trastuzumab and lapatinib (not by either agent alone), and that anti-HER family targeting TKIs induce compensatory increase of HER3 expression (Sergina et al., 2007; Baselga et al., 2012 c; Grøvdal et al., 2012), are clearly in line with this.

Deregulation of acetylation and deacetylation of core nucleosomal histones play an important role in aberrant gene expression in human cancers (Archer and Hodin, 1999; Kouzarides, 1999). Histone deacetylases (HDACs) are evaluated as molecular targets. A variety of HDAC inhibitors (HDACi) are studied as potential anticancer agents. HDACi impact a large number of molecules which may include HER family members. One of those is entinostat (SNDX-275), which targets, among others, HER2 and HER3 (Huang et al., 2009). Entinostat preferentially inhibits cell proliferation/survival and inactivates downstream signaling in HER2 overexpressing breast cancer cells compared with breast
cancer cells with basal HER2 levels. It reduces the levels of HER2, HER3, pHER2, pHER3, p-AKT and p-MAPK. In contrast, entinostat has little effect on EGFR. Entinostat combined with trastuzumab exhibits synergistic antiproliferative activities in the breast cancer cell lines SKBR3 and BT474 (Huang et al., 2011). Additionally, the combination of entinostat and erlotinib or lapatinib is active in breast, lung and HNSCC cell lines, which are resistant to either drug when administered as single agent (Witta et al., 2009).

Another potential strategy against HER3 signaling is the use of heat shock protein 90 (HSP90) inhibitors. HSPs facilitate the conformational maturation and refolding of a variety of signaling proteins during environmental stress, including EGFR, HER2 and HER3, as well as downstream signaling components, such as RAF1 and AKT1. They can reduce the stability of HERs and cause abrogation of HER signaling (Baselga and Swain, 2009). One of these HSP90 inhibitors is 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) that binds to a highly conserved pocket in the HSP90 chaperone protein and thereby inhibits its function. Inhibition of HSP90 with 17-AAG leads to a downregulation of HER2 and HER3 in LNCaP and LAPC-4 prostate cancer cells and in human prostate cancer xenografts (Solit et al., 2002). A combination of trastuzumab and 17-AAG had some efficacy in HER2-positive metastatic breast cancer patients whose disease had previously progressed on trastuzumab (Modi et al., 2011). The study in 27 patients revealed an overall response rate of 22% and a clinical benefit rate (complete response + partial response + stable disease) of 59% with a median duration of response of 147 days. Preclinical studies in BT-474 human breast cancer xenografts have suggested that client proteins rebound within 24 to 72 hours. Therefore, more frequent administrations of HSP90 inhibitors might be necessary (Solit et al., 2003).

**Targeting HER3 ligands**

Blocking HRG by using HRG antisense cDNA inhibits tumorigenicity and metastasis of the breast cancer cell line MDA-MB-231 in vivo (Tsai et al., 2003). Therefore, HER3 ligands could be potential therapeutic targets. Theoretically, mAbs could be directed to HER3 ligands such as HRG, preventing them from stimulating HER3. However, at present, such antibodies are not available.

**BIOMARKERS FOR EFFECT OF HER3-TARGETING**

In most cases, the tumor-promoting functions of HER3 are not harnessed through amplifications or mutations, making it more difficult to identify patients with HER3-dependent tumors. Therefore, biomarkers might be useful for prediction and monitoring of treatment effects, as well as supporting decision making during clinical development and clinical practice. With all above mentioned HER3-targeting agents there is as yet still uncertainty about biomarkers (Şendur et al., 2012).
At this point, a critical role of HER3 has been confirmed in HER2-amplified breast cancers, and pertuzumab demonstrates this relevance. Therefore, HER3 protein expression might identify patients that are more likely to respond to HER3 targeting agents. HER3 immunostaining was part of a larger panel of biomarkers in the NeoSphere biomarker analysis (Gianni et al., 2011). More than 300 primary breast cancer samples from this neo-adjuvant trial (Gianni et al., 2012) were assessed for association between protein expression measured immunohistochemically and mRNA expression and pathological complete response. Preliminary data showed that HER2 staining predicted for trastuzumab/pertuzumab benefit ($P = 0.001$). HER3 staining did not correlate with treatment outcome. Protein levels of HER2, HER3, PTEN, and pAKT and mRNA expression of EGFR, HER2, HER3, amphiregulin and betacellulin in tumor tissue were also assessed in the phase III CLEOPATRA trial (Baselga et al., 2012 b; Gianni et al., 2012). Patients with high HER2 protein ($P = 0.05$), HER2 mRNA ($P = 0.008$) or HER3 mRNA ($P = 0.03$) had a better prognosis. In addition to HER3 mRNA being a biomarker for pertuzumab effect, HER3 protein quantification may also be candidate biomarker for trastuzumab response. High HER3 protein expression associated with poor clinical outcomes (Lipton et al., 2013).

Presence of HER3 is very dynamic due to its compensatory role in HER signaling, particularly in response to HER2, HER3 and PI3K pathway inhibition, which may attenuate the antitumor action of these inhibitors (Makhija et al., 2010; Serra et al., 2011; Chandarlapaty et al., 2011). Combining HER3 with HER2 inhibitors enhanced tumor growth inhibition, in vivo (Garrett et al., 2013a). This might imply a potential role for a more dynamic assessment of HER3 tumor status than tumor staining. Molecular imaging techniques allowing whole body imaging of molecular tumor characteristics can potentially address this. $^{111}$Indium (In)-trastuzumab and $^{89}$Zirconium (Zr)-trastuzumab can visualize HER2 overexpressing lesions with SPECT and PET, respectively (Perik et al., 2006; Gaykema et al., 2012). The possibility of assessing the drug treatment effects of targeting HER2 such as HSP90 inhibitors, lapatinib and trastuzumab by quantifying the (change in) radioactive signal of radiolabeled antibodies against HER2, has already been investigated in vivo (Oude Munnink et al., 2010; Oude Munnink et al., 2012; Schröder et al., 2011; McLarty et al., 2009). These drugs reduced the tumor uptake of the radiolabeled tracers.

For the assessment of HER3 tumor status, the 64Copper (Cu) labeled AMG 888 (U3-1287) using the chelate DOTA ($^{64}$Cu-DOTA-U3-1287) was studied in mice bearing HER3 overexpressing BxPC3 human pancreatic cancer xenografts (Sharp et al., 2011). MicroPET imaging of the radiolabeled antibody showed significant contrast between the tumor and non-target tissues and increased tracer blockade with increasing doses of
non-radioactive AMG 888. $^{64}$Cu-DOTA-U3-1287 is currently evaluated in subjects with advanced solid tumors (NCT01479023). Another antibody which has been used for molecular imaging in vivo is the $^{89}$Zr-labeled GE-huMab-HER3 antibody. This antibody is specifically taken up in HER3 overexpressing FaDu HNSCC and H441 lung cancer xenografts (Terwisscha van Scheltinga et al., 2010) and is currently tested in an ongoing clinical trial. In this trial patients receive one or two doses of $^{89}$Zr-labeled GE-huMab-HER3 in addition to unlabeled GE-huMab-HER3 to evaluate the in vivo biodistribution and organ pharmacokinetics of GE-huMab-HER3 (NCT01482377). In addition, a novel $^{111}$In-labeled bispecific radioimmunoconjugate consisting of trastuzumab Fab against HER2 and HRG against HER3 has been used to image tumors in athymic mice, that express one or both receptors, using small-animal SPECT/CT. This antibody showed in vitro specific binding to tumor cells displaying HER2 or HER3 and was taken up specifically in vivo in xenografts expressing one or both receptors. Bispecific antibodies could be useful for imaging HER2/HER3 heterodimers because their bivalent properties may result in preferential binding to the heterodimerized forms (Razumienko et al., 2012).

A caveat of assessing HER3 itself, in addition to its dynamics, is that it may not reflect the signaling status after dimerization. Therefore, the assessment of pHER3 might also be of interest. The HER3 antibody GE-huMab-HER3 showed a dose-dependent effect on tumor growth inhibition in FaDu xenograft models together with a decrease of pHER3 and HER3 and an even bigger decrease in the pHER3/HER3 ratio (Meneses-Lorente et al., 2012). This pHER3/HER3 ratio might be useful as a pharmacodynamics marker. For prediction of trastuzumab and pertuzumab effect, the NeoSphere and the CLEOPATRA biomarker analyses have not reported pHER3 data yet (Gianni et al., 2011; Baselga et al., 2012b). Therefore, the place of pHER3 immunohistochemistry on human tumor tissue, as biomarker for anti-HER3-targeting agents, is at present unknown. In addition, proximity-based immunoassays have been developed for profiling HER3 activation in formalin-fixed, paraffin-embedded breast tumor samples. These assays can quantitatively measure HER2 total, HER3 total, HER2/HER3 heterodimers, pHER3 and HER3/PI3K kinase protein expression. They are currently being investigated preclinically to define mechanism of drug action, and in clinical trials to determine their value in predicting tumor response to targeted therapies (Wallweber et al., 2013).

Quantification of HRG mRNA may be a surrogate for paracrine or autocrine activation of HER3. In patients with HNSCC, high HRG mRNA expression in tumors with both paracrine and autocrine phenotypes was associated with pHER3, possibly indicating active HER3 signaling (Shames et al., 2013). Moreover, in a phase I trial of MEHD7495A, the two patients who showed partial responses had HNSCC tumors that expressed high levels of HRG (Cervantes-Ruiperez et al., 2012). Elevated HRG expression and activated HER3 were
also strongly associated with lapatinib sensitivity in non-HER2 amplified cancer cells, especially in HNSCC (Wilson et al., 2011). Therefore, HRG could represent a biomarker for a subset of HNSCC patients that can respond to HER3 targeting therapies.

HER3 mRNA levels in tumors might be a potential biomarker for tumor growth inhibition mediated by HER3 signal inhibition. HER3 mRNA expression was only upregulated in xenograft models that were responsive to the anti-HER3 therapy with GE-huMab-HER3 (Pickl et al., 2012). This suggests that these tumors, which are dependent on HER3 signaling, might counter-act HER3 target inhibition by upregulating mRNA levels. In platinum-resistant ovarian cancer, the clinical benefit of pertuzumab therapy appears to increase at lower HER3 mRNA levels (Makhija et al., 2010). Low HER3 mRNA levels also correlated with a favorable clinical outcome in metastatic HER2-positive breast cancer patients treated with trastuzumab in combination with pertuzumab (Cortes et al., 2012). Ligand activation of the HER3:HER2 complex leads to downregulation of HER3 mRNA, in vitro (Makhija et al., 2010). Therefore, low mRNA expression may be an alternative biomarker for active HER3 signaling and might predict response to HER3-targeting. However, in the CLEOPATRA trial, patients with high HER3 mRNA, who were treated with pertuzumab plus trastuzumab plus docetaxel, had a better prognosis (Baselga et al., 2012 b). HER3 mRNA in patients with HER2-positive breast cancer did not predict treatment response to pertuzumab in the NeoSphere trial (Şendur et al., 2012; Gianni et al., 2011).

CONCLUSIONS AND FUTURE PERSPECTIVES
In this review, we have described the way in which HER3 acts as partner in crime with the other HER family members. Its role, particularly in resistance to agents targeting other HER members, clearly provides a rationale for targeting HER3 itself. The dynamics of HER3 have supported the concept of targeting more than one HER family member at once for the most optimal anti-tumor effect. This concept is now increasingly adopted into clinical designs. However, targeting HER3, the smartest brother of the HER family with its lacking intrinsic tyrosine kinase activity and its compensating role in HER signaling, remains a particular challenge. Various compounds that block HER3 (antibodies) or interfere with HER3 dimer signaling (antibodies, TKIs, HSP90 inhibitors) are currently in clinical development. Preclinically, these compounds have shown promising results. If these HER3-targeting agents are proven to have clinical antitumor activity, hopefully biomarkers can support patient selection. Biomarker candidates such as HER3 protein and mRNA expression have already been included in biomarker analyses. However, obtaining the optimal biomarker(s) for prediction of anti-HER3-targeting treatment effect will still require considerable effort in the near future. Rigorous inclusion of comprehensive panels of these biomarkers in prospective clinical trials should provide
more insight in the complexity of HER3-targeting, which will eventually support the best biomarker for future clinical use.

**CONFLICT OF INTEREST**
The authors declare that there are no conflicts of interest.

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