Targeting the Human Epidermal Growth Factor Receptors with Immuno-PET: Imaging Biomarkers from Bench to Bedside

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ABSTRACT

Human epidermal growth factor receptors (HER) are targeted by a growing number of inhibitors which directly block the receptors on HER-expressing tumor cells or interfere with their signaling pathways. However, HER-expression is variable between tumors and resistance against these drugs is a well-known clinical problem necessitating further research to optimize therapeutic regimes for HER-positive patients. Currently, information about the potential biomarker status is routinely obtained *ex vivo* from biopsy specimens. HER-targeted imaging biomarkers on the other hand, could measure *in vivo* the receptor expression across the entire disease burden and help to monitor early responses to treatment. This review describes current status of HER-specific imaging agents with a particular focus on moving these molecular probes from early preclinical studies to clinical trials.

EGF RECEPTORS AS VALID IMAGING TARGETS

The human epidermal growth factor family (HER) of receptor tyrosine kinases has been the focus of intense translational research over the past three decades. The HER family includes four members: EGFR (HER1, ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). These structurally related proteins coordinate a complex signaling network that plays a crucial role in the development and evolution of cancer (Fig.1). There are at least eleven different ligands (e.g. epidermal growth factor (EGF), transforming growth factor (TGFα), neuregulins (NRGs) that are known to bind to these receptors. Although all four receptors have the same essential domains, the functional activity of each domain varies. HER2 does not have a known ligand, but exists in the extended 'active' conformation state, constitutively available for dimerization with the other three members. In contrast, HER3 can bind to several ligands but lacks fully intrinsic tyrosine kinase activity as it is unable to bind adenosine triphosphate. EGFR and HER4 have active tyrosine kinase domains and known ligands (*1*). Ligand binding triggers intracellular signaling through a tightly controlled network of downstream signaling mechanisms, which drive and regulate many cellular processes. The two main signaling pathways, which are utilized by all HER receptors, are the Ras/MAPK (mitogen-activated protein kinase) that leads to cellular proliferation, differentiation, as well as migration, and the PI3K (phosphatidylinositol3-kinase)/Akt that primarily serves to promote cell proliferation and survival through progression of the cell cycle and inhibition of apoptosis (*2*). Changes in the regulation of receptor activity may cause uncontrolled cell growth and result in the initiation and maintenance of several solid tumors, including breast, gastric, ovarian, and non-small cell lung cancers. Cancer patients whose tumors have HER alterations (e.g. receptor overexpression, mutation or gene amplification) tend to have more aggressive tumor behavior. Furthermore, in the majority of cases receptor-positivity is associated with resistance to certain types of chemotherapy, hormone therapy and ionizing radiation, consequently leading to a shorter time to disease progression and worse patient survival (*2*).

These findings have led to the development and widespread implementation of specific HER inhibitors, including antibodies targeting the extracellular domain and small molecules that prevent phosphorylation of the intracellular domain (tyrosine kinase inhibitors; TKI). Several of these targeted drugs have been approved for clinical use including a recombinant humanized version of an anti-HER2 monoclonal antibody (mAb), trastuzumab (Herceptin®), which has shown significant clinical efficacy in HER2-overexpressing metastatic breast cancers when combined with chemotherapy. The success of trastuzumab was followed by the introduction of other therapeutic antibodies and antibody-drug conjugates (ADC) targeting the HER2, *e.g.* pertuzumab (Perjeta®) and the ADC trastuzumab-emtansine (T-DM1, Kadcyla ®). Among the small molecular inhibitors, lapatinib (Tykerb ®) has shown activity in HER2-positive cancers that have relapsed following trastuzumab treatment. Gefinitib (Iressa®) and erlotinib (Tarceva®) are further examples of TK inhibitors, which act on the epidermal growth factor receptor. The chimeric anti-EGFR monoclonal antibody cetuximab (Erbitux®) is used for the treatment of metastatic colorectal cancer, metastatic non-small cell lung cancer and head and neck cancer.

Even though these targeted therapies have improved the treatment outcome in the early stage of cancer, many tumors still manifest intrinsic resistance and even those that initially respond to the treatment develop resistance within a year.

The receptor status thus represents a major factor in selecting patients for targeted therapies, therefore more precise assessment of the HER family expression levels and

activation of downstream signaling pathways would prove beneficial in identifying new treatment paradigms.

Currently, HER-specific alterations (e.g. protein overexpression, receptor mutation, translocation, and epigenetics changes) can be analyzed by methods such as: immunohistochemistry (IHC), quantitative polymerase chain reaction, proteomics, next-generation sequencing of the tumor tissues derived postoperatively or via biopsies. These techniques aid in our understanding how cancer cells adapt to the treatment and become resistant to it. However, these are invasive methods prone to sampling errors and most likely confounded by inter- and intratumor heterogeneity of receptor expression within analyzed biopsy specimens. Moreover, collecting sequential biopsies to monitor changes in receptor expression over time in response to treatment is not only practically difficult and ethically challenging, but also it will not reflect the presence of the resistant clones in multiple metastatic sites. Therefore, developing and validating non-invasive specific imaging biomarkers capable of confirming the presence of accessible targets early in the disease progression may help to resolve the dilemmas in which information about protein status is essential when considering a new treatment regimen and the biopsy is impractical.

Positron emission tomography (PET) using radiolabeled mAbs, antibody fragments or engineered protein scaffolds (immuno-PET), has the potential to offer non-invasive criteria able to identify the presence and accessibility of the target, to measure more accurately tumor response in a timely fashion (e.g. immediately following treatment initiation), as well as to assist in patient stratification. Furthermore, immuno-PET can provide information about the heterogeneity of both target expression and therapeutic response, which are increasingly recognized as key factors in therapeutic resistance, especially in patients with advanced disease where target expression may vary from site to site and biopsy of a single site may not represent the entire burden of a disease. The non-invasive nature of immuno-PET also allows for performing multiple imaging sessions to adequately assess treatment response by longitudinal studies.

Although introduction of immuno-PET into routine clinical practice may add some complexity and increase costs of studies, with the appropriate use it has the potential to assess the efficacy of novel anti-cancer compounds during the early stages of development. It will lead to minimizing the investment wasted by taking ineffective treatments through further clinical testing. Noteworthy, in 2012 Pfizer announced that 43% of their Phase II trials failed due to a negative outcome at clinical proof-of-concept studies, where the candidate-drug mechanism of action was not adequately tested in an earlier phase of development (*3*).

ROLE OF IMAGING HER RECEPTORS IN PATIENT STRATIFICATION AND MONITORING THERAPY RESPONSE

With the increasing number of therapeutic antibody candidates targeting members of the HER family there is a need to better understand their biochemical/biological properties. Recently, several research groups have intensively worked on the development of imaging probes targeting these receptors, since the information about their expression helps to identify those patients who are likely to benefit from anti-HER targeted therapies. However, only a few studies have explored the relationship between imaging, genomics, and histopathology, therefore there is still a need for larger prospective studies to elucidate whether these methods can play a complementary role in assessing target expression and if their combination may prove useful.

A growing spectrum of radionuclides with longer half-life has recently become available for conjugation with mAbs. The most frequently used are ^{124}I ($t_{½}$ = 4.17 days), ^{64}Cu ($t_{½}$ = 12.7 h) and, ^{89}Zr ($t_{½}$ = 78.4 h). Depending on the radioisotope, different labeling methods are used. Iodine can be directly attached to mAb through the simple and widely available procedures, whereas radiometals are introduced indirectly by first conjugating the suitable chelator to the mAb and then non-covalently binding the metal ion. Among the radiometals ^{89}Zr is the isotope of choice for mAbs that become internalized upon binding to its target since it becomes trapped (residualized) inside the cell and its physical half-life matches well the relatively slow pharmacokinetics of mAbs in solid tumors.

Furthermore, it can be chelated by DFO under mild conditions which do not affect mAb structure. But slow clearance of intact mAbs requires a prolonged interval from injection to scan and one of the principal concerns regarding the use of ^{89}Zr in the clinic is the substantial radiation dose received by patients compared to shorter-lived nuclides. Indeed, it has been reported for example that ^{89}Zr-trastuzumab (37 MBq; 18 mSv) leads to 2.5-fold higher radiation exposure when compared to conventional ^{18}F-FDG PET (37 MBq, ~7 mSv) (*4*). These considerations have prompted the development of alternative approaches such as engineered mAb fragments, diabodies, and affibodies that still have high specificity and affinity of the potential therapeutic mAb, but their molecular weight below 60 kDa allows for rapid renal clearance and elimination. The clearance time of these targeting agents also matches the favorable short half-life of ^{18}F ($t_{½}$ = 109.8 min) allowing for same day imaging.

Many preclinical small animal studies have been performed with ^{89}Zr-labelled anti-HER radioligands to determine their tumor targeting characteristics. Knowledge obtained in these studies has made the translation of these agents into clinical trials possible. Typical examples of such practice comes from breast cancer studies where the development of HER2-targeted therapies was one of the most significant breakthroughs in the treatment of HER2-positive patients leading to a marked increase in therapeutic response. However, due to disease heterogeneity, the reported discordance between HER2 status in primary tumor and metastasis measured by IHC has been very high (13-30%) indicating the need for introducing immuno-PET imaging into clinical practice. Figure 2 shows a typical example of ^{89}Zr-labeled trastuzumab PET/CT in a patient with metastatic breast cancer.

An interesting early example is a study presented by Dijkers et al. in HER2-positive breast cancer patients (*4*). The authors demonstrated high accumulation of ^{89}Zr-trastuzumab in HER2-positive lesions in the liver, lung and bone. Additionally, unknown metastases were detected across a locally compromised blood-brain barrier. Also, ^{64}Cu-DOTA-trastuzumab has shown promise in early clinical trials (*5*). In another study, Smith-Jones et al. have shown that ^{68}Ga-labelled Fab fragments detect different levels of HER2 receptors in preclinical models (*6*) and this approach has already demonstrated its feasibility in an early clinical study (*7*). Besides, preclinical imaging using ^{89}Zr-trastuzumab has also been used to monitor a decrease in HER2 expression in response to drugs targeting Hsp90, a chaperone protein that plays an important role in mediating HER2 expression. Interestingly, these studies have recently been translated to early-stage human trials, where ^{89}Zr-trastuzumab imaging was used to measure changes in HER2 expression in response to the Hsp90 inhibitor NVP-AUY922 (*8*). Furthermore, it is well known that for T-DM1 to be active, the presence of an intact HER2 receptor is of pivotal importance since the internalization of the cytotoxic moiety of the drug depends on the binding of trastuzumab to the external domain of HER2. Gebhart et al., at the 2014 ASCO meeting, presented data showing that the presence or absence of HER2, as indicated by the ^{89}Zr-trastuzumab uptake and a response, as indicated by decrease in ^{18}F-FDG uptake provided an essentially 100% accurate prediction

of later clinical response to HER2-directed therapy with T-DM1 administration in metastatic HER2 positive breast cancer (NCT01565200 clinical trial) (*9*). This trial is a great example indicating that ^{89}Zr-trastuzumab is an accurate non-invasive test capable of identifying non-responding patients prior to TDM1 administration. Recently, several groups including ours have also tested the feasibility of using affibody molecules specifically targeting HER receptors (*10*). In 2010 the first clinical studies using ^{68}Ga and ^{111}In-labelled Affibody-based HER2 imaging agents were performed (DOTA[0]-Z [HER2:342-pep2, ABY-002, ABY-025). Both compounds showed specific tumor uptake and allowed for high contrast imaging of HER2-positive tissues (*11,12*). Combined, these results further strengthen the applicability and promising future of HER2 imaging as routine clinical practice.

^{89}Zr-labelled anti-EGFR mAb cetuximab has also been investigated but most studies to date have been carried out in animal models. Several groups have shown that receptor expression level alone is not sufficient to predict patient response to anti-EGFR therapies and that there is no correlation between tracer uptake and protein level evaluated by IHC (*13*). Other studies have demonstrated that the majority of tumors responding to EGFR kinase inhibitors harbor activating mutations in the EGFR kinase domain (*14*) suggesting that imaging of EGFR mutant expression could be more useful in selecting the right patient population for personalized treatment. On the other hand, Chang et al. have recently shown that the uptake of EGFR-targeted ^{89}Zr-panitumumab correlated well with the EGFR expression (*15*), and van Dijk et al. have presented that ^{111}In-cetuximab-F(ab')$_2$ can be used to monitor the effects of EGFR inhibition combined with irradiation in head and neck carcinoma models (*16*). Also, comprehensive reviews on PET imaging of EGFR expression and multimodality imaging the HER-kinase axis in cancer have been published elsewhere (*17,18*). Similarly to HER2- and EGFR-PET, the very recent preclinical and clinical data emphasizes the potential of HER3-imaging. This receptor is the only member of the family that lacks fully catalytic kinase activity and accordingly its activation following heregulin binding is inherently dependent on heterodimerization with other members of the EGFR family. Beyond that, unlike other HER receptors, HER3 has 14 tyrosines in the C-terminal tail; six of them are docking sites directly recruiting the p85 subunit of PI3 kinase. As a consequence, HER3 is a key node in the activation of the PI3K/AKT pathway, promoting tumor cell survival, proliferation, and metastasis (*19*). Recent studies have highlighted HER3 as a fundamental signaling receptor involved in the establishment of malignancy with several groups demonstrating that HER3 overexpression correlates with advanced disease stage and decreased overall survival (*20*). In addition, emerging evidence from studies of EGFR- and HER2-driven cancers have shown that these cancers invariably become resistant to anti-HER therapies, indicating that HER3 mediates in their treatment failures (*21*). For example, it has been demonstrated that there is a strong association between sensitivity to TK inhibitors including gefitinib and erlotinib and HER3 inactivation (*22,23*). These observations suggest that up-regulation of HER3 receptors might be an indication of poor prognosis, where agents targeting HER3 could potentially provide a novel approach towards the treatment of such cancers.

Since HER3 lacks enzymatic activity, efforts have been put towards the development of mAbs targeting the receptor ectodomain and inhibiting various mechanisms by which HER3 can become activated, with some being alrady in phase I and II clinical trials (i.e. MM-121, U3-1287, and LJM716) (*24,25*). The preclinical findings that have been presented so far with these mAbs appear to be very promising. or example, the study of MM-121 combined with the anti-EGFR mAb cetuximab in a mouse model of lung cancer clearly showed that resistance to cetuximab is due to HER3 ctivation, and blocking HER3 signaling by adding

MM-121 into treatment combination led to a greater and more durable response (*26*). Moreover, LJM716 has shown significant growth inhibitory effects in HER2-positive cells and xenograft models when combined with trastuzumab or cetuximab (*27*).

However, the activity of HER3 selective drugs in humans has not been robustly demonstrated and the overall data may be viewed as modest. Recently, Patritumab (U3-1287) was used in combination with erlotinib in NSCLC patients who had progressed after at least one course of chemotherapy. The authors reported that the efficacy of the combination was encouraging, but there was no clear correlation between tumor response and HER3 expression in tumor tissues or serum soluble HER3 levels. The lack of correlation could be due to the type of tumor tissues that were used (an archived tissue from the initial diagnosis) or the relatively low number of patients enrolled in the study (*28*). Therefore, the absence of a suitable biomarker to evaluate HER3 status still remains the major hurdle in the development of HER3-targeted therapies and effective patient selection. Consideration has been given to markers established for overexpression of partner kinases, such as HER2 or markers for the expression level of ligands that activate HER3 (e.g. NRG-1). For example, Schoeberl et al. have shown that the level of NRG-1 can be a better biomarker than receptor expression for the selection of patients that may benefit from MM-121 treatment (*26*). But even though NRG-1 is the most potent inducer of HER3 phosphorylation, using it as an effective biomarker might be quite limiting due to the existence of multiple routes of HER3 activation, such as mutation, ligand-independent dimerization and potential contribution in receptor activation from other HER family ligands.

In light of these findings, there is clearly a need for the development of imaging biomarkers that could guide the clinical use of innovative anti-HER3 targeted therapies. So far, to assess the HER3 tumor status, ^{89}Zr radiolabelled mAb (GSK2849330) is being evaluated in patients with advanced solid tumors (clinical trial; NCT12345174).

In addition, a novel ^{111}In-labelled bispecific agent consisting of a trastuzumab Fab fragment targeting HER2 and NRG-1 targeting HER3 has been shown using small-animal SPECT/CT to specifically accumulate in tumor xenografts where HER2 is co-expressed with HER3 (*29*).

Recently, we have developed a ^{89}Zr-labelled Affibody$_{HER3:8698}$ recognizing the HER3 antigen (Fig.3). High tracer accumulation was found in human breast cancer MCF-7 and BT-474 (HER3+++) xenografts but not in MDA-MB-231 tumors (HER3+) confirming the binding specificity of the probe. Similar high contrast images between targeted and non-targeted tissues were obtained for ^{89}Zr-RG7116, an anti-HER3 antibody (*30*), but it required 144 hours for the mAb to be cleared, whereas the ^{89}Zr-Affibody$_{HER3:8698}$ provided images within the 1-3 h post injection. Furthermore, when mice bearing MCF-7 tumors were treated with AUY922, an Hsp90 inhibitor that is known to induce proteasome mediated degradation of phosporylated HER2 and HER3, there was a significantly higher tumor uptake of ^{89}Zr-Affibody$_{HER3:8698}$ in mice receiving the drug than in the control animals. This unexpected enhancement in HER3 expression following the treatment has been found to correlate with the recovery of HER3 and subsequent upregulation of the insulin-like growth factor 1 (IGF-1) receptor and activation of the PI3K/AKT pathway (data on file). This data underlines the potential of a HER3 imaging agent as a tool for not only patient stratification for HER3 targeted therapies but, more importantly, also for monitoring receptor level during therapeutic interventions.

CONSIDERATIONS AND FUTURE PERSPECTIVES

The observations that HERs are key players in the establishment of malignancy have led to a number of mAbs and T inhibitors, blocking EGFR and HER2, resulting in clinically meaningful anti-cancer activity. Continuously growing evidence points to HER3 upregulation as an important factor in resistance to HER-targeted therapies. The number of anti-HER3 antibodies currently under development and tested in the clinical trials further underscores the importance of this target. Experimental and clinical evidence suggest that the dynamics of HER3 force to combine agents that directly block HER3 with compounds that interfere with receptor dimer signaling. Specific imaging agents could provide unique information as cancer biomarkers, including quantification of receptor heterogeneity and early detection of changes in target expression in response to therapy in parallel to high throughput sequencing technologies.

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Figure 1. EGF receptors and their major activation pathways.

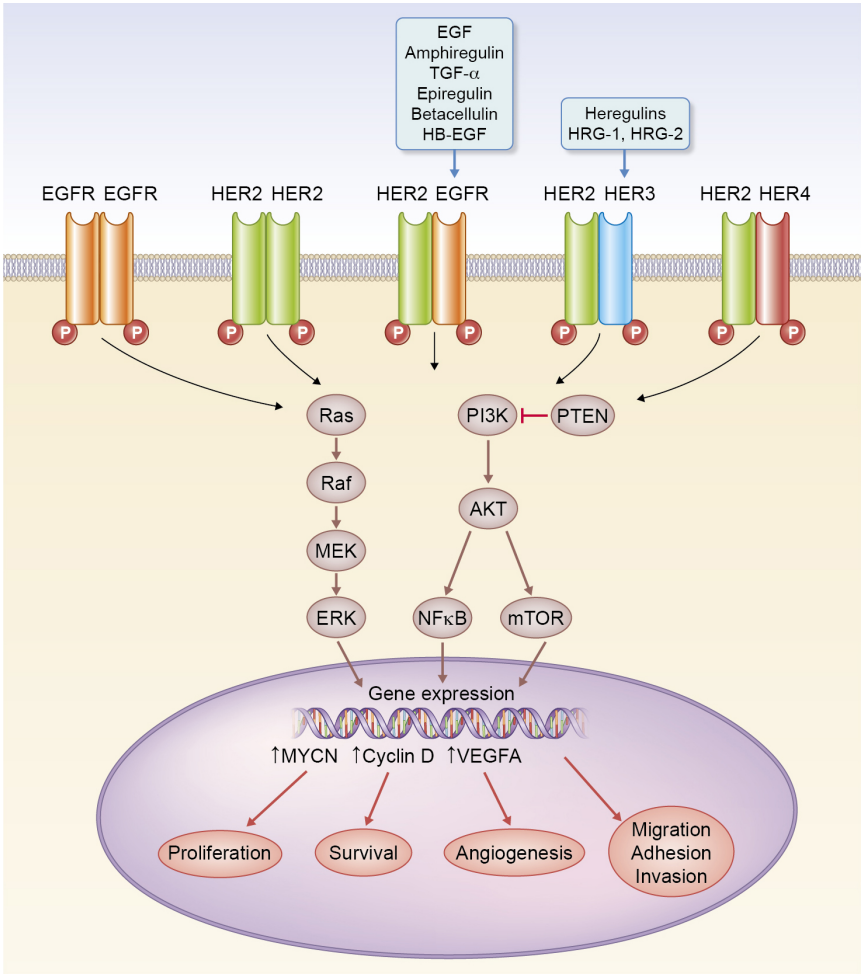

Figure 2. Whole body PET (A) and transaxial PET/CT (B-D) 96 hours after intravenous injection of 37 MBq ^{89}Zr-trastuzumab in a patient with HER2-postive breast cancer metastatic to the liver (blue arrow), lymph nodes (red *) and bone (green *).
Images courtesy of Dr. Udai Banerji, Institute of Cancer Research, London, UK

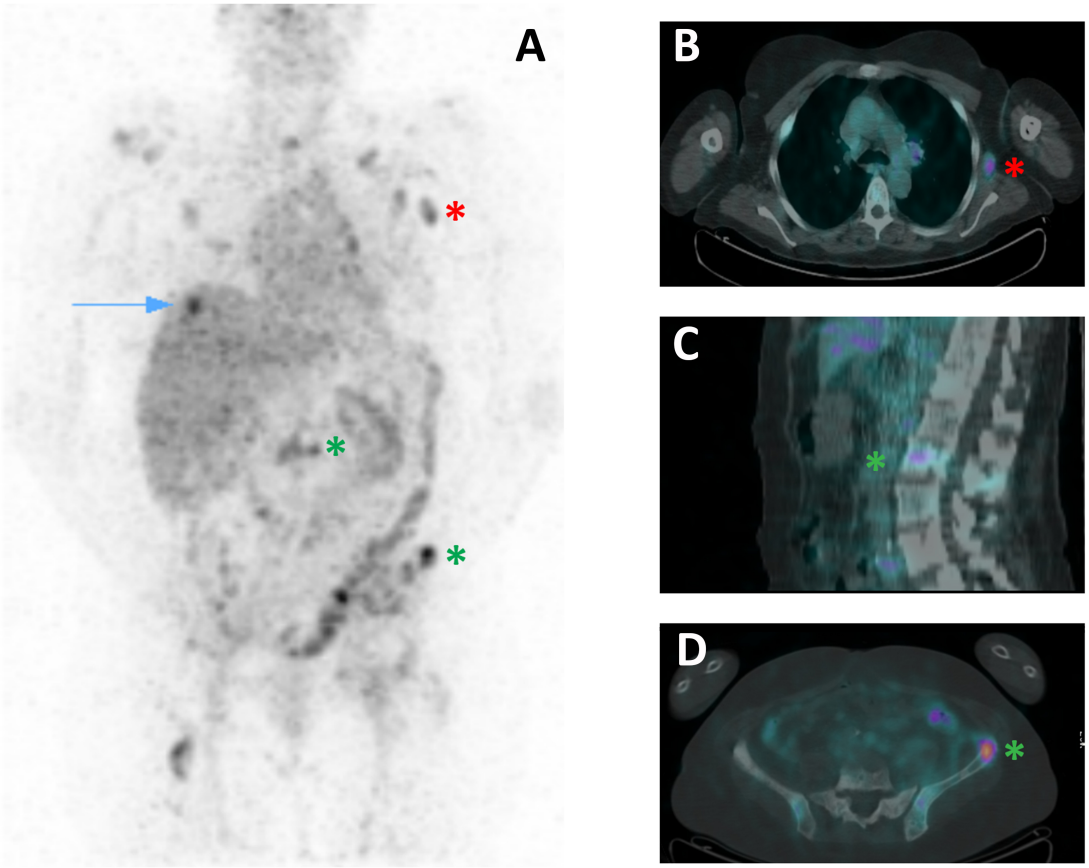

Figure 3. A. ^{89}Zr-Affibody$_{HER3:8698}$ uptake 3 h post injection in mouse bearing MCF-7 tumor. B. Ex vivo HER3 immunohistochemistry staining of MCF-7 tumor showing distribution of HER3 receptors at different tumor regions. C. Confocal microscopy image of HER3-positive MCF-7 cells exposed to Affibody$_{HER3:8698}$-DyLight-633. D. Diagram illustrating potential routes of inhibiting HER2/HER3 pathway activation and imaging the receptor status.