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# CLINICAL INVESTIGATION

# HER2/neu: an increasingly important therapeutic target. Part 1: basic biology & therapeutic armamentarium

This is the first of a comprehensive three-part review of the foundation for and therapeutic targeting of HER2/neu. No biological molecule in oncology has been more extensively or more successfully targeted than HER2/neu. This review will summarize the pertinent biology of HER2/neu and the EGF receptor family to which it belongs, with attention to the biological foundation for the design and clinical development of the entire range of HER2/neu-targeted therapies, including efforts to mitigate resistance mechanisms. In conjunction with the subsequent two parts (HER2/neu tissue expression and current HER2/neu-targeted therapeutics), this comprehensive survey will identify opportunities and promising areas for future evaluation of HER2/neu-targeted therapies, highlighting the importance of HER2/neu as an increasingly important therapeutic target.

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The history of the molecule known as HER2/neu dates back to the earliest studies of virus-associated oncogenes. In 1979, studies of avian erythroblastosis virus identified two putative viral oncogenes, v-erbA and v-erbB [1–3]. Subsequently, the Bishop group identified the normal avian (chicken, 'c') and mammalian homologs (c-erbA and c-erbB) [4,5], and that the encoded protein is a membrane glycoprotein [6]. This gene and its gene product were rapidly recognized to have a relationship to the recently identified, EGF receptor (EGFR) [7,8]. Simultaneous, but separate, studies in the Weinberg laboratory in chemical-induced rat tumors identified an oncogene – neu – from a rat neuroblastoma that could transform wild-type 3T3 cells. This oncogene was noted to have tyrosine kinase activity and sequence homology with c-erbB and the EGFR genes [9–12]. This relationship became clearer with the studies of Semba *et al.* in which the individual genes and the gene nomenclature for EGFR family members (c-erbB1 = EGFR,

c-erbB2 = neu, c-erbB3 = EGFR-3, and eventually, c-erbB4 = EGFR-4) were established [13]. The fact that the neu molecule and coding sequence was originally identified in the rat species and only recently has a biological function been demonstrated for the murine gene locus [14–16] has complicated studies of the biology and fueled a separate nomenclature in humans; HER1, 2, 3 and 4 (Table 1). The high degree of homology between HER2 and rat neu (~85%) along with the recognition that the protein product of the *c-erbB2* gene was known in various circles as HER2 or as neu led to the commonly used terminology HER2/neu.

The structural elements (Figure 1 & Table 1) and the dimerization patterns (Figure 2) result in a complex biology for the HER family. These type 1 transmembrane proteins contain, in the extracellular portion of the molecule, three cysteine-rich Furin-like domains and two L domains. Each L domain consists of a right hand, single-stranded β-helix, which forms the ligand-binding site. It

| Gene nomenclature | EGFR nomenclature | HER nomenclature | Common protein nomenclature | Kinase signaling capacity | Ligand(s) |
|---|---|---|---|---|---|
| *erb-B1* | EGFR | HER1 | EGFR | Active | EGFR, amphiregulin, TGF-β, epigen, β-cellulin, HB-EGF |
| *erb-B2* | EGFR-2 | HER2/neu | HER2/neu | Active | |
| *erb-B3* | EGFR-3 | HER3 | EGFR-3 | Inactive | Neuregulin 1, Neuregulin 2 |
| *erb-B4* | EGFR-4 | HER4 | EGFR-4 | Active | Neuregulin 1, Neuregulin 2, Neuregulin 3, Neuregulin 4, β-cellulin, HB-EGF |

EGFR: EGF receptor.

remains unclear whether the variable arrangement of the three Furin-like domains has any biological significance. The intracellular domain contains a protein tyrosine kinase consensus domain and multiple phosphorylation sites that permit downstream signal transduction in all but HER3, which has an in active protein tyrosine kinase domain [17]. Receptor dimerization, as a homodimer or heterodimer, is essential for signal transduction and involves a portion of the transmembrane domain along with a portion of the membrane proximal, extracellular domain.

Although the ligands for each HER family member are relatively restricted, some ligands engage more than one HER family member (Figure 2 & Table 1) [18]. The ligands for HER1 (EGFR) include: EGF, amphiregulin, TGF-α and epigen [19,20]. Three ligands can bind to either HER1 or HER4 including: β-cellulin, epiregulin and HB-EGF. The remaining identified HER family ligands are members of the Neuregulin family. Neuregulin 1 and Neuregulin 2 both have α- and β-isoforms. Neuregulin 1 (aka Heregulin, NDF) and Neuregulin 2, both with α- and β-isoforms, can bind to HER3 or HER4, while Neuregulin 3 and Neuregulin 4 can only bind HER4. Importantly, HER2 has no established ligand.

Two facets of HER family biology limit the potential combinatorial diversity. HER3 is a dead kinase [17] and thus, any signaling that occurs is dependent upon the dimerizing partner for HER3. HER2 has no defined ligand and, thus, is dependent upon the dimerizing partner for ligand-dependent signaling. Recent structural studies suggest that ligand interaction with extracellular domains I and III result in alterations in domain II that permit heterodimerization [21,22], the relative activity of anti-HER2 antibodies (trastuzumab and pertuzumab) are related to binding to different domains. HER2 exists in a structurally receptive conformation that allows it to readily form dimers and heterodimers [23] and is the preferred dimerization partner for heterodimers involving HER1, HER3 and HER4. This conformation favors heterodimerization with HER2 in lieu of homodimerization where presumably two ligands would be required to convert two proteins into the permissive dimerization conformation [21,22]. Phosphorylation events occurring on the cytoplasmic tails of the HER proteins, either via auto- or trans-phosphorylation [24–26], are essential for signal transduction.

Signaling through the HER family members involves a limited number of major signal transduction pathways (Figure 2) [18,27]. The PI3K/AKT/mTOR pathway is the major pathway involved. Additional pathways include the ERK, the Ras/Raf, the Rho/Rac and the phospholipase C pathways [27–29]. An appreciation of the complexity of these signaling pathways is essential for understanding the resistance mechanisms that come into play to counteract therapies targeting the HER molecules, including HER2/neu [30–32]. These pathways impact a number of biological processes (Figure 2). Signaling through the HER family members has been demonstrated to influence the regulation of proliferation, transcription, autophagy, apoptosis and chemotaxis. Crosstalk between the various involved pathways results in more extensive propagation of the ligand-induced signal [18]. The overexpression of HER2/neu, with its intact kinase activity that is not dependent upon engagement of a distinct cognate ligand, is believed to perturb the balance of signaling within the HER family and contribute to dysregulated growth [33].

The HER2/neu molecule, which will be the focus of the remainder of this review, is expressed in a wide

range of normal tissues, overexpressed in a variety of tumor types, with or without gene amplification, and is an established target for antitumor therapeutics. The normal expression of HER2/neu appears to be primarily transcriptionally regulated [34–36]. A number of transcriptional elements and factors have been identi-fied, which vary between normal and malignant trans-formed states and by tissue [35,36]. In the absence of gene amplification, transcription dysregulation is one mechanism for tumor overexpression of HER2/neu [37,38]. Post-transcriptional regulation of HER2/neu expression has also been described [39–41]. Recent work
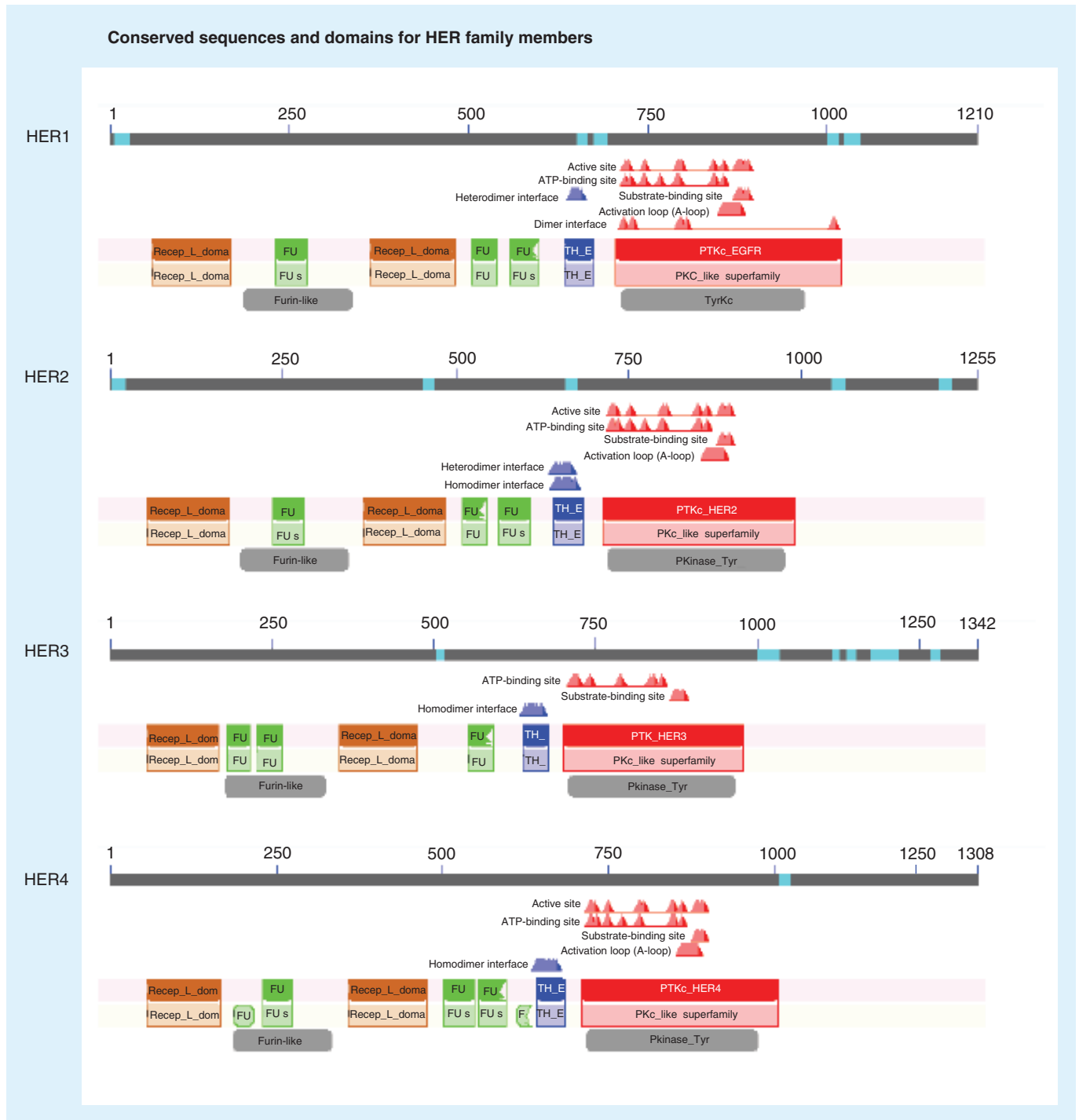


**Figure 1. Protein and coding sequences for HER1, HER2, HER3 and HER4 were retrieved from the US National Center for Biotechnology Information Entrez Gene database and submitted for evaluation in the context of the Conserved Domain Data Base at the NIH.** (Summary graphic output for each HER molecule has been combined into this figure for comparative purposes).
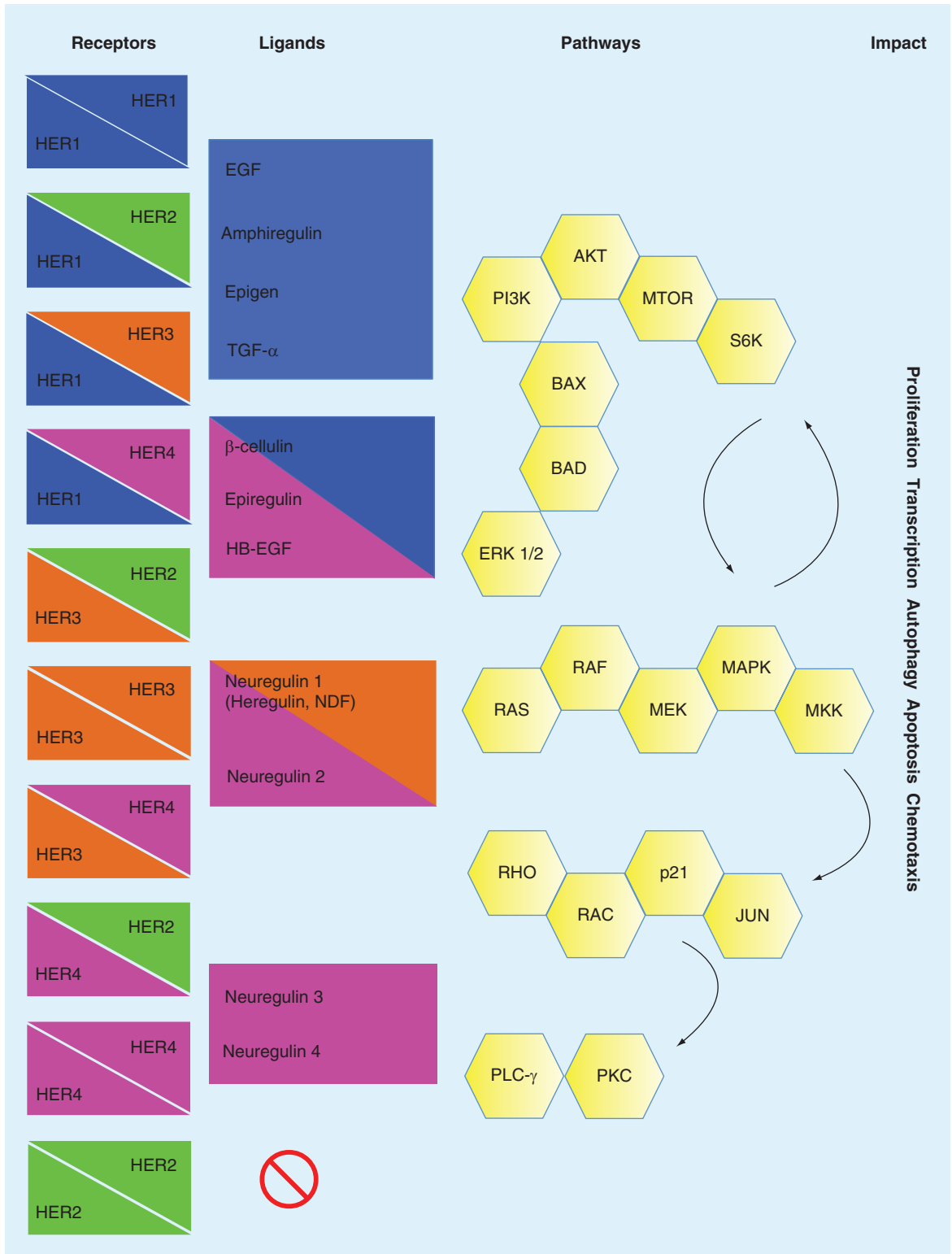
**Figure 2. The individual HER heterodimers are depicted in the left hand column and their cognate ligands color coded for binding to their respective HER molecules.** Blue: HER1 and its respective ligands; green: HER2; orange: HER3 and its respective ligands; fuschia: HER4 and its respective ligands. Ligands with two colors bind to both HER molecules. The major engaged signaling pathways are depicted with arrows indicating well-established nodes for 'cross-talk'. The major biological impacts of HER family-mediated signaling are depicted on the right.

supports an important role for miRNAs in the biology and expression of HER2/neu [42–45]. Glucocorticoids have been reported to stabilize HER2/neu mRNA levels [39] and HSP90 stabilizes the expression levels of at least HER1 and HER2 by inhibiting their degradation via the ubiquitin proteasome pathway and by facilitating dimerization [46,47]. Thus, there are transcriptional and other nonclassical post-transcriptional mechanisms by which tumor overexpression of HER2/neu may occur in the absence of gene amplification. The exemplary work by Slamon *et al.* demonstrated that overexpression of HER2/neu by gene amplification in breast cancer imparted a poor prognosis [48] and was subsequently independently confirmed by Berger *et al.* [49]. HER2/neu overexpression without gene amplification has also been associated with prognosis in multiple tumor types, potentially related to the transcriptional dysregulation or other mechanisms.

Evidence for a critical biological function of HER2 has been derived from gene-targeting studies in mice, which occurred nearly 20 years after the original description of neu. Either constitutive kinase dead or *erbB2*-null mice demonstrate embryonic lethality secondary to gross cardiovascular abnormalities, most significantly abnormal development of the heart [14,16]. The *erbB2*-null mice or perinatal cre/lox deletion of erbB2 also revealed an important role for development and maintenance of the nervous system [14–16]. The fact that major defects in erbB2 compromised mice were somewhat limited, despite HER2 being the preferred dimerization partner for the HER family members, amplifies the complexity and redundancy present within the EGFR family, but also provides a foundation for understanding the unexpected cardiac toxicity experienced with initial therapeutics directed at HER2/neu.

## HER2/neu-targeted therapeutics

Development of therapeutic strategies targeting HER2/neu have been driven by the underlying biology of the EGFR/HER family and HER2/neu, with a major focus on breast cancer [50]. Antibodies directed at the extracellular domain were generated [51], with both agonist and antagonistic properties [33,52–54]. The absence of a cognate ligand for HER2/neu precluded development of either ligand-clearing antibodies or small-molecule decoy ligands or ligand-binding site inhibitors. However, with the launch of the small-molecule targeted therapy era, small-molecule tyrosine kinase inhibitors (TKIs) have been developed and approved for HER2/neu and other HER family members. Antitumor antigen-specific immunotherapeutic strategies (tumor vaccines) and modulators of HER2/neu expression, such as inhibitors of HSP90, are advancing through clinical studies and have shown promise. Future strategies targeting HER2/neu may well employ new small-molecule pathway inhibitors or combinations of HER2/neu therapeutic strategies or agents to minimize the development of resistance [55].

## Antibody-based therapies
### Trastuzumab (Herceptin®)

The clinical and commercial success of the anti-HER2/neu antibody trastuzumab (Herceptin®) is self-evident and establishes without question the proof-of-concept for therapeutic targeting of HER2/neu. The series of studies leading to the approval and broad application of this monoclonal antibody have been held up as a prototype for bench-to-bedside translation [56]. Initial studies characterizing the *erbB2* gene in human tumors demonstrated that it was amplified in breast [48] and subsequently in ovarian adenocarcinoma [57]. Because HER2/neu was a known cell-surface molecule and because antagonistic antibodies had been reported, the development of an antagonistic antibody for clinical application was logical, given the state of the art in the late 1980s [58,59]. The characterization of the antibody designated 4D5 demonstrated significant growth inhibition of SKBR3 breast carcinoma cells, in which the *erbB2* gene is amplified, and sensitized them to cytotoxic effects of TNF-$\alpha$ [58] resulting in the initial patent (US Patent No 5,677,171). The epitope recognized by the 4D5 antibody resides within extracellular domain IV of HER2/neu [60], can elicit antibody-dependent cellular cytotoxicity, and importantly, disrupts HER2/neu-mediated signaling, with modest disruption of dimerization. Interestingly, this disruption is much more pronounced for the HER1:HER2 heterodimer than the HER2:HER3 heterodimer, even though HER3 is the preferred dimerization partner for HER2 [61]. This antibody would go on to be genetically engineered to create a chimeric, 'humanized' IgG1subclass monoclonal antibody [62], and take its place in the clinical armamentarium as trastuzumab (Herceptin) [63]. In the landmark clinical studies, associated cardiac toxicity was observed, particularly in patients previously treated with anthracyclines [64]. The aforementioned murine work identified the critical role for HER2/neu in cardiovascular development [14,16], wherein *erbB2*-null animals have an embryonic lethal phenotype secondary to abnormal heart development [14], and was reported only slightly before this unexpected toxicity was observed, in 27% of study participants.

The development of resistance and tumor progression on treatment was observed in the initial studies of trastuzumab in the advanced disease setting. Resistance can be generally classified as primary resistance

or as acquired/secondary resistance [65]. Given the complexity of the EGFR family and the extensive crosstalk between signaling pathways, it comes as no surprise that the mechanisms of trastuzumab resistance are diverse and likely to be multiple in any one patient [31,65,66]. Perhaps the easiest mechanism to understand arises from the recognition that the extracellular domain of HER2/neu can be proteolytically cleaved into a soluble form, can be detected in the circulation, and that levels of this soluble form have been variably associated with disease burden, prognosis and inversely with response to treatment [67–70]. Indirect studies suggest that the soluble ECD of HER2 could both act as a sink for trastuzumab [71,72] and may elicit or be associated with anti-HER immune responses, particularly antibody responses [73]. There are data supporting that the remaining portion of the cleaved HER2/neu molecule has increased endogenous constitutive signaling capacity [74], suggesting an additional mechanism of resistance. Additionally, an alternatively spliced form of HER2/neu has been described that is truncated, yielding a potentially secreted form of the molecule, but which appears to be sequestered in the perinuclear cytoplasm [75]; however, a role for this potential alternative fragment in normal or neoplastic tissues has not been documented. Interestingly, binding of trastuzumab to HER2/neu has been reported to inhibit the proteolytic cleavage of the extracellular domain [76,77]. Although there is no compelling evidence to suggest that HER2/neu undergoes mutation to abrogate the trustuzumab-recognized epitope, there is data that MUC-4 can mask the binding site and may be responsible for some primary resistance [78]. Perturbations of downstream signaling pathways can also yield resistance (Figure 2). Loss or low levels of expression of PTEN have been associated with a decreased response to trastuzumab, perhaps through release of the counter-regulatory input of PTEN on AKT [79,80]. Activating mutations of PI3K have also been associated with trastuzumab resistance [81,82], as has increased Rac activity [83,84]. There are also data to suggest that increased expression of other HER family members can overcome the trastuzumab-mediated inhibition of HER2/neu function, and thereby confer resistance [85]. Other receptor kinases can also function in a similar manner to HER2/neu and other HER family members, including IGF-1 receptor, Met and erythropoietin receptor [86–90].

## Pertuzumab

In the initial screen that identified 4D5 as a potent therapeutic antibody for HER2/neu gene-amplified tumors, another antibody with a nonoverlapping epitope, 2C4, was identified [59]. In contrast to trastuzumab, this antibody binds to the extracellular domain II, resulting in enhanced steric blocking of dimerization via binding to the requisite domain for dimerization and signaling [91]. Thus, the humanized 2C4 antibody (pertuzumab) [92] is more broadly effective at inhibiting heterodimer formation with HER2/neu. Pertuzumab has activity with lower levels of expression of HER2/neu [93] and unlike trastuzumab, which has limited ability to inhibit heterodimerization between HER2/neu and HER3; pertuzumab inhibits signaling by HER3 ligands [94]. Pertuzumab may inhibit, but not abrogate HER2/neu HER3 heterodimerization and may have less activity inhibiting HER2/neu HER1 heterodimerization [95,96]. It is unclear if pertuzumab interferes with the heterodimerization of HER2/neu with alternate tyrosine kinase receptors such as IGF-1R, and Met.

## Trastuzumab emtansine (T-DM1; Kadcyla®)

An alternate strategy for improving the efficacy of trastuzumab, in the setting of lower expression of HER2/neu or resistance to trastuzumab, is the conjugation of a cellular toxin or radioisotope in a manner analogous to that demonstrated for hematologic malignancies; Adcetris®, Zevalin®, Bexxar® and Mylotarg®. Trastuzumab emtansine (T-DM1) is a conjugate of trastuzumab and the cellular cytotoxic agent mertansine (DM1) that is a derivative of the microtubule disruptive macrolide maytansine, which, when conjugated to the trastuzumab antibody using the linking agent 4-(3-mercapto-2,5-dioxo-1-pyrrolidinylmethyl)-cylohexanecarboxylic acid, is known as emtansine [97]. The use of this linking agent substantially improved the release of mertansine by proteolytic cleavage once internalized, relative to more traditional reducible disulfide-linking chemistries that were used in the early studies of antibody conjugates of this cytotoxic agent [98,99]. Mertansine binds to tubulin at a site different from that of the vinca alkaloids and is substantially more potent at inhibiting microtubule formation [97]. Given the observed cardiac toxicity with trastuzumab, a more potent agent potentially targeting lower level HER2/neu expression might be expected to have greater cardiotoxicity, but early pharmacokinetic data suggested that this is not the case [100].

Other modified antibodies targeting HER2/neu have been designed for both therapeutics and imaging, although many suffer from the limitations of traditional disulfide-based conjugation chemistries [99]. These conjugates include affitoxin [101], pseudomonas exotoxin [102,103], ricin A chain [104], various probes (radioactive and nonradioactive) [104–112], photosensitizers [113–115], RNAse [116,117], cytokines [118] or chemotherapeutic agents [119–122]. A Phase II study of a novel

PET agent (zr89-labeled trastuzumab) as a predictor of response to trastuzumab emtansine (ZEPHIR) has recently been initiated in Europe (NCT01565200). Additionally, bispecific antibodies targeting HER2/neu have been developed [123–127] taking advantage of the intrinsic immunologic function of antibodies recognizing HER2/neu. Finally, aptamers selected for recognition and binding to the HER2/neu molecule can deliver cytotoxic agents to HER2/neu-positive cells as an alternative to antibody-based delivery [128].

## Tyrosine kinase inhibitors

The groundbreaking work that identified Imatinib as an effective small-molecule inhibitor of the tyrosine kinase activity of the oncogene-derived fusion protein BCR/Abl launched the era of molecularly targeted therapeutics [129]. The appreciation of the signaling capacity and tyrosine kinase activity of HER2/neu made identification of small-molecule inhibitors an obvious priority, ultimately resulting in the development of lapatinib ditosylate and other targeted therapeutics [130–134]. An additional impetus for the development of this class of therapeutic agent was the observed increased incidence of CNS metastases in the setting of breast cancer patients treated with trastuzumab or other antibody-based HER2/neu-targeted therapies, with this often being the site of progression while other systemic disease remained under control. Well-designed small molecules might have better CNS penetration and potentially circumvent this problem. Generalizing from the Imatinib experience, it was anticipated that multiple TKIs would be needed as acquired mutations could lead to resistance. Although all TKIs are designed to inhibit receptor signaling, there are differences in spectrum, reversibility and potential secondary mechanisms [135]. Recent observations that many of these TKIs interact with various drug transporters including ABCB1, ABCG2 and the P-glycoprotein multidrug resistance transporter [136–138] in a semi-selective and dose-dependent manner, adds another complicating element to their development and clinical application.

The first TKI developed to target HER2/neu was lapatinib ditosylate (GW572016, Tykerb), which is a reversible inhibitor of both HER2/neu and HER1/EGFR [139]. In the initial Phase I studies of lapatinib ditosylate the toxicities matched those predicted from clinical experience with HER1/EGFR inhibitors erlotinib and gifitinib; rash, fatigue and gastrointestinal symptoms (diarrhea) [140–144]. Interestingly, no additional or unexpected cardiotoxicities were observed when lapatinib ditosylate was combined with trastuzumab in early-phase trials [141–144]. The clinical efficacy observed in several Phase II studies and a

landmark Phase III trial led to US FDA approval of lapatinib ditosylate [145]. Whether this represents additional or more effective targeting of HER2/neu versus the addition of HER1/EGFR inhibition remains to be determined. Either would be expected to have clinical impact given the mechanisms of resistance to trastuzumab and the crosstalk between HER family member signaling pathways described above. Recently, acquired mutations in HER2/neu have been identified in association with resistance to lapatinib ditosylate [146] suggesting that, as in the case of imatinib, other inhibitors targeting the same molecule will be necessary to treat those tumors with acquired resistance. An additional concern arises from the observation that lapatinib ditosylate increases the shedding of HER2/neu [147], a potential mechanism for resistance particularly to HER2/neu-directed antibody-based therapies.

Other TKIs targeting HER2/neu have been generated and are at various stages of development. In contrast to lapatinib ditosylate, which is a reversible inhibitor of HER1 and HER2, the pipeline of TKIs targeting HER2/neu contains second-generation irreversible inhibitors [148], which can be categorized as either pan-HER or dual HER1/HER2 inhibitors. These TKIs, like essentially all drugs in this class, are oral, multikinase inhibitors inhibiting HER2/neu, other members of the HER family, and other receptor tyrosine kinases (Table 2).

Afatinib (Gilotrif, BIBW 2992, Tomtovok) is in the class of irreversible TKIs that target HER2/neu along with other HER family members. This 'second generation' of TKIs with irreversible inhibitory activity are felt to not only have increased potency, but also the capacity to inhibit tumors that have progressed on the first-generation reversible inhibitors, typically by acquiring mutations that confer resistance to first generation TKIs [132,148,149]. The majority of the effort in development of afatinib has been directed toward its activity inhibiting EGFR (HER1), but it was rapidly recognized to also inhibit HER2/neu [150,151]. Preclinical work also suggested that afatinib would be synergistic with radiation therapy [151,152]. Pharmacokinetic [150,153] and Phase I studies have led to a number of administration schedules and doses with acceptable toxicities including: 2 weeks with daily administration of 70 mg with 2 weeks off [154]; continuous 50 mg daily dosage [155,156]; continuous 40 mg daily dosage [157]; 3 weeks of daily 40 mg and 1 week off [158]; afatinib 90 mg days 2–4 in combination with paclitaxel 75 mg/m$^2$ on day 1 every 3 weeks [159]; and, more multidrug regimens containing cisplatin and either paclitaxel or 5-fluorouracil at 20 or 39 mg daily, respectively [160]. The dose-limiting toxicities are, as expected from the first-generation HER family-targeting TKIs,

Table 2. Tyrosine kinase inhibitors with HER2/neu inhibitory activity.

| Tyrosine kinase inhibitor | Reversibility | Documented receptor targets† | Clinical trial status‡ | Tumor types§ |
|---|---|---|---|---|
| Gifitinib | Reversible | **EGFR** (HER1), HER2/neu | Approved (1) | Multiple |
| Erlotinib | Reversible | **EGFR** (HER1), HER2/neu | US FDA approved (2) | Mulitiple |
| Lapatinib | Reversible | **HER2/neu**, EGFR (HER1) | FDA approved (3) | Breast |
| TAK-285 | Reversible | **HER2/neu**, EGFR (HER1) | Phase I | Multiple |
| Afatinib | Irreversible | **EGFR** (HER1), HER2/neu, HER4 | FDA approved (4) | Lung (NSCLC), breast, colorectal |
| Neratinib | Irreversible | **HER2/neu**, EGFR (HER1) | Phase III | Breast, lung (NSCLC), colon |
| Pelitinib | Irreversible | **EGFR** (HER1), HER2/neu | Phase II (no Phase III registered) | Lung (NSCLC), colorectal |
| AST1306 | Irreversible | **HER2/neu**, EGFR (HER1) | Preclinical only | |
| Canertinib | Irreversible | **EGFR** (HER1), HER2, HER4 | Phase II (no Phase III registered) | Breast, lung (NSCLC), ovarian |
| Dacomitinib | Irreversible | **EGFR** (HER1), HER2, HER4 | Phase II (no Phase III registered) | Lung (NSCLC), head and neck squamous cell and glioblastoma |
| BMS-599626 | Irreversible | **EGFR** (HER1), HER2, HER4 | Phase I (no Phase II or III registered) | HER2-positive tumors |
| BMS-690514 | Reversible | **EGFR** (HER1), HER2, VEGFR-1, 2 and 3 | Phase II (no Phase III registered) | Breast, lung (NSCLC) |
| AEE788 | Reversible | **EGFR** (HER1), **HER2**, VEGFR-1 and 2 | Phase I (no Phase II or III registered) | Multiple, brain (GBM) |

†Target molecules in bold represent the primary molecular target for the respective tyrosine kinase inhibitor.
‡(1): approved outside of the USA, for first-line lung NSCLC, with mutated EGFR; (2): first-, second- and third-line lung NSCLC, with mutated EGFR; first line for advanced pancreatic adenocarcinoma; (3): second-line advanced or metasatic HER2/neu⁺ breast adenocarcinoma; (4) first-, second- and third-line lung NSCLC, with mutated EGFR.
§Tumor types that have been evaluated in either Phase II or III clinical studies with the exception of TAK-285, BMS599626 and AEE788.
EGFR: EGF receptor; GBM: Glioblastoma multiforme; NSLC: Non-small-cell lung cancer.

diarrhea and rash. Afatinib was given fast track status for NSCLC by the FDA [161] leading to recent FDA approval. Afatinib has been reported to inhibit HER4 [162], which would indicate that it resides in the class of irreversible pan-HER inhibitors. Other members of this class of pan-HER irreversible TKIs include canertinib (CI-1033), which has TKI activity across the entire HER family, HER1, HER2 and HER4 and was noted to have radiosensitizing activity [163,164]. Phase I studies conducted by different groups using different schedules including oral continuous, oral for 14 days with 7 off, oral for 7 days with 14 off, and intravenous, came to entirely different maximum-tolerated doses [165–170] with dose-limiting toxicities being rash, nausea, diarrhea or fatigue depending, in part, on schedule and

route. Dacomitinib (PF-00299804) is a second-generation irreversible pan-HER TKI related to canertinib, but with better pharmacokinetic and bioavailability properties [171,172]. Although HER2/neu is subject to inhibition by this agent, the overwhelming bulk of its development has focused on its inhibition of EGFR (HER1) [171–173]. In Phase I studies, dacomitinib did not appear to have the same variability in maximum-tolerated dose, established at 60 mg daily, by schedule and route as in the first-generation pan-HER inhibitor canertinib [174–176]. Dacomitinib, at 45 mg daily the dose carried forward to Phase II studies, is able to inhibit HER family members with acquired mutations that confer resistance, particularly to erlotinib and gefitinib, but amplification of the mutated HER sequence

can lead to dacomitinib resistance [177]. BMS599626 (AC480) is also a second-generation pan-HER inhibitor [178,179] that appears to be a radiosensitizing agent [179]. Somewhat surprisingly, the dose-limiting toxicities were not the usual mucocutaneous toxicities, which did occur, but rather QT prolongation and elevation of liver transaminases with a maximum-tolerated dose of 600 mg daily [180].

A subset of pan-HER inhibitors were designed to include significant inhibitory activity of one or more of the VEGFRs. AEE788 is a pan-HER inhibitor with capacity to inhibit the three VEGF receptors, which has roughly equivalent activity inhibiting HER1 and HER2 [181]. Preclinical work with AEE788 suggests antiangiogenesis activity, radiosensitizing capacity, and potential synergy with aromatase inhibition in breast cancer models [182,183]. The initial Phase I study demonstrated the expected dose-limiting toxicities rash and diarrhea with the maximum-tolerated dose of 450 mg/day, but based on biomarker data suggested that in humans there was limited antiangiogenic activity [184]. BMS-690514, is another pan-HER inhibitor that includes inhibitory activity for all three VEGF receptors [185]. Pharmacokinetic and Phase I studies have been reported identifying the maximum-tolerated dose of 200 mg daily [186–190]. In contrast to AEE788, BMS-690514 appears to have significant anti-angiogenic activity by toxicity profile and biomarker analyses [186,191]. There is also evidence for synergism with radiation when BMS-690514 is administered in sequence with radiation [192].

Neratitib (HKI-272, WAY-179272) is an irreversible, dual EGFR and HER2 TKI moving through the development pipeline [193]. The initial Phase I study identified a maximum-tolerated dose of 320 mg daily, with the expected dose-limiting toxicity being gastrointestinal (diarrhea). In subsequent Phase I and II studies, conducted in breast and lung adenocarcinoma, this toxicity proved to be excessive resulting in a dose of 240 mg daily being carried forward into more advanced clinical studies [194–197]. Pelitinib (EKB-569) is another analog of established dual HER1 and HER2 inhibitors [198,199]. Preclinical and Phase I studies suggest that pelitinib has greater activity in inhibiting EGFR/HER1, is capable of overcoming resistance to other EGFR-targeted TKIs due to acquired mutations, and also inhibits signaling through HER2/neu [198–200]. Phase I studies have been reported establishing a maximum-tolerated dose of 75 mg/day with gastrointestinal toxicity (diarrhea) being limiting [200–204]. AST1306 is another recently developed dual HER1 and HER2 TKI, which has yet to progress to early-phase clinical studies [205]. Several other dual targeting TKIs have been developed and entered into clinical studies but abandoned, including: MP-412 (AV-412), XL647, CP-724,714 and PPI-166 [206].

## HER2/neu antigen-specific immunotherapy

The work of Slamon *et al.* in defining the critical role for HER2/neu gene amplification in identifying a group of breast cancer patients with particularly poor prognosis [48], the awareness of the role of HER2 in receptor signaling for all HER molecules [18], and the clinical success of an exogenous antibody recognizing HER2/neu (trastuzumab) [63] made HER2/neu an appealing target for development of antigen-specific, antitumor vaccines [207,208]. The high degree of homology with other receptor tyrosine kinases, including other members of the HER family, and the expression of HER2/neu in normal tissues, pose significant challenges that need to be addressed, including overcoming the anticipated immunological tolerance while maintaining specificity of the elicited immune response. Much of the preclinical work was performed in HER2/neu transgenic mouse models due, in part, to the fact that a murine homolog was not confirmed until the early 2000s [14–16], although other models were also employed (dog, guinea pig, rat, primate) [209–214]. The majority of these have focused on the extracellular domain of HER2/neu due, in large part to the high degree of homology of the intracellular kinase domain with other receptor tyrosine kinases both in and outside of the HER family [209,215–219], although some strategies have included elements from the intracellular domain [213–215]. Indeed, cytotoxic T-lymphocytes directed against HER2/neu have been documented to react with HER3 and HER4 [220].

In the mid-1990s, preclinical work demonstrated a number of potential peptides derived from HER2/neu that could be viable target antigens giving rise to peptide-based immunotherapy strategies [221–223]. These moved into early-phase clinical studies in the first decade of the 2000s including: several early-phase clinical studies of the immunodominant E75 peptide with granulocyte macrophage colony-stimulating factor as a biological adjuvant (now referred to as Neu-Vax™) [221–223]; of an improved E75 vaccine, AE37 (NCT00524277) [224–227]; and of the subdominant G2 peptide (NCT00524277) [228–230]. Other groups have ongoing Phase I and II clinical studies examining other peptides, other adjuvants and/or combinations of immunomodulatory agents (NCT00058526, NCT00194714, NCT00791037, NCT00952692, NCT01355393, NCT01376505 and NCT01632332). Early data suggest synergy between peptide-based anti-HER2/neu vaccine and other HER2/neu-targeted therapies, for example trastuzumab [231,232] and lapatinib ditosylate [233]. However, in some cases,

there is substantial toxicity arising from combining or incorporated adjunctive elements [221].

A multitude of other vaccination strategies and immunotherapeutic methodologies have been evaluated in both preclinical and early clinical studies [207,208]. Dendritic cells (DCs) are the most potent antigen presenting and immune-stimulating cells within the immune system, thus, DC-based immunotherapeutic strategies directed against HER2/neu have been investigated, including DCs loaded with various fragments or peptides from the HER2/neu sequence [234–237] (NCT00266110 and NCT00923143) and transduced DCs [213,237–243] including adenoviral transduced autologous DCs expressing the extracellular and transmembrane domains of HER2/neu (NCT01730118). A preparation analogous to Sipuleucel-T (the first FDA-approved cellular based-immunotherapy) [244] is being developed, Lapuleucel-T, consisting of a HER2/neu truncated fusion with granulocyte macrophage colony-stimulating factor that is used to generate an autologous mixture of antigen-presenting cells targeting the HER2/neu component [245].

HER2/neu has been targeted using polynucleotide or DNA vaccine strategies involving both syngeneic and xenogeneic sequences, the latter to improve the magnitude of elicited immune responses [246–254], in prime boost strategies combining DNA vaccines with viral vector-based vaccines [255] and with gene-modified allogeneic cellular vaccines (NCT00095862) [256]. Viral vector vaccine strategies using adenovirus vectors [257–260], alphavirus vectors [212,213,261], vaccinia virus vectors (NCT00485277 and NCT01152398), vesicular stomatitis virus [262] and polyoma virus systems [263,264] have all been used to elicit anti-HER2/neu immune responses. The intracellular bacteria *Listeria monocytogenes* has also been adapted as an immunotherapeutic vector system and studied in strategies to elicit anti-HER2/neu immune responses [215,265].

The broad strategy of adoptive cellular therapy has also been investigated. Typically, this has involved clones of autologous T-lymphocytes derived from tumor-infiltrating lymphocytes or from peripheral circulation [266]. Some groups have combined peptide vaccination, in an effort to increase the number of circulating HER2/neu reactive T-lymphocytes, to provide an alternative source of T-lymphocytes for expansion and adoptive transfer back to the patient (NCT00791037). An alternative approach to generate HER2/neu-reactive T-lymphocytes is to genetically modify autologous lymphocytes with chimeric antigen receptors (CARs) [267,268]. The history of CAR strategies for targeting HER2/neu is quite substantial. Soon after the identification of antibodies that recognized HER2/neu, they were leveraged to

try to target T-lymphocytes to HER2/neu-expressing tumors [269–271]. Initial efforts involved electroporation of a murine T-cell hybridoma with cDNA-encoding antigen-binding domain from a HER2/neu-recognizing antibody and the CD3 ζ-chain transmembrane and intracellular signaling domain [269]. Although this provided proof-of-principle for imparting functional capacity to T-lymphocytes enabling them to recognize and respond to the HER2/neu antigen, electroporation and the use of T-cell hybridomas was not readily translatable to the clinical arena. The development of retrovirus/lentivirus vectors provided for more efficient transduction of T-lymphocytes and NK cells [272–280]. Various groups have sought to refine the CAR constructs, including tuning affinity of the HER2/neu antigen-recognition domain [275], utilizing the T-cell receptor peptide recognition domain instead of an antibody-based recognition domain [281] and incorporation of costimulatory elements [282] within the construct. With the identification of Heregulin as a ligand for heterodimers involving HER3 or HER4, including those with HER2/neu, a chimeric ligand molecule (heregulin and intracellular signaling component from the CD3 ζ-chain) was developed [283]. Novel transduction methods [284] and the application of allogeneic CAR-modified cells [285], in a bone marrow transplant-like setting, have been developed. Within the antitumor immunotherapy community there is enthusiasm for the potential of CARs to overcome some of the major hurdles encountered in the clinical application of adoptive cellular therapy [286–290], due in large part to the work of Carl June with CAR-modified T-lymphocytes in the setting of chronic lymphocytic leukemia [291]. However, recent data on the adoptive transfer of CAR engineered T-lymphocytes has raised some concerns as to toxicity and potential differences between targeting cell surface molecules on hematopoietic versus solid tumor cells [286,292].

## Other modulators of HER2 expression or activity

Less direct targeting of HER2 has been explored. Since the early 2000s, inhibition of HSP90 has been documented to inhibit HER2 and HER1-mediated cell signaling, along with increasing ubiquitin-mediated proteosomal degradation of HER2/neu [46,47]. The diverse activities and clients of HSP90 result in a number of biological pathways being impacted by HSP90 inhibition [47], several of which involve, directly or indirectly, the HER2/neu signaling pathways. Thus, it is not surprising that a substantial effort is being put forth by numerous groups and companies to develop HSP90 inhibitors with attractive therapeutic to toxicity ratios and pharmacokinetics [293–304]. One of the more impor-

tant activities associated with HSP90 inhibition is the reversal of resistance to both trastuzumab and hormonal agents [305,306]. Importantly, there is preclinical data indicating that HSP90 inhibitors are synergistic with both trastuzumab [307–309] and lapatinib ditosylate [309,310]. Phase I and II clinical studies of first-generation HSP90 inhibitors have been published [311–313]. As with most biologic or targeted therapies, the effectiveness is quite schedule-dependent. Another compound, tephrosin, can downregulate both HER1 and HER2 expression [314], providing an alternative pathway to HSP90 for the downregulation of HER2/neu expression. There are scattered studies of 'natural products' and pharmacologic agents that modulate signaling or expression of HER2 [314–317]. Preclinical studies in a murine breast cancer model of an adenovirus encoding kinase dead HER2/neu have been conducted as a potential mechanism to downregulate HER family signaling and take advantage of the promiscuity of heterodimerization with HER2/neu [318]; based on the biology of the HER family it is predicted that this strategy would have its greatest effect in tumors with high HER3 expression. The role of these pathway modulators remains to be determined, but the HSP90 inhibitors in particular are showing substantial promise and have the potential to circumvent resistance to other therapeutic agents.

With the clinical success of immune checkpoint inhibitors targeting CTLA-4 and the PD1 pathway [319] in melanoma, the incorporation of other adjunctive maneuvers to enhance tumor immunogenicity and immunotherapeutic efficacy [320] are indicated for HER2/neu-directed immunotherapeutic strategies. The co-targeting of other biological processes, such as the autophagy pathway, or other signaling pathways, such as the HedgeHog pathway, may be beneficial and could be simultaneously impacted by agents such as the HSP90 inhibitors [321–323]. Similarly targeting the microenvironment in which tumors survive may be a complimentary approach [324,325]. There are recent data that support a role for HER2/neu in the biology of cancer stem cells, which opens the potential for application of HER2/neu-targeted therapy in combination with therapeutic strategies targeting the cancer stem cell population [326].

## Conclusion

The breadth of potential therapeutic agents targeting HER2/neu far exceeds that directed at any other tumor-associated, biological molecule. The development of this repertoire is driven in large part by the fact that HER2/neu is overexpressed in a broad range of tumor types and the biological role HER2/neu plays in the broader HER family signaling network. The ongoing characterization of the biology of HER2/neu and other HER family members continues to provide critical insight into mechanisms of resistance to individual therapeutic agents or strategies and also illuminates novel targetable nodes within the HER2/neu signaling network. Although FDA approval of antibodies, antibody conjugates, and TKIs targeting HER2/neu has been obtained for breast, gastric and esophageal adenocarcinomas, there are other clear opportunities in other tumor types. There is reason to be encouraged that other therapeutic approaches to targeting HER2/neu will find a place in the therapeutic armamentarium, including immunotherapeutic strategies and modulators of HER2/neu expression such as the HSP90 inhibitors. The basic science, preclinical and clinical work reviewed above substantiates the proposition that targeting HER2/neu has and continues to be the consummate translational research success story and making HER2/neu an increasingly important therapeutic target.

## Future perspective

Our increasing appreciation of the complexity of intracellular signaling pathways, particularly with respect to crosstalk between pathways and the impact of other nonsignaling biological pathways, is dramatically driving the field towards systems biology and network analyses. The basic laboratory work elucidating these biological interactions that began well over a decade ago, including those involving the HER signaling pathways, is now poised to make an impact in the clinical arena in the next decade. Although there will still be a role for determining HER2/neu overexpression, at the protein and genetic level, network analyses from expression profiling (proteomic or genomic) of individual tumors will reveal both resistance mechanisms and identify potential nodes for combining other agents to improve the efficacy of targeting HER2/neu. It is quite possible, given recent and ongoing technical advances, that tissue obtained from small needle biopsies will be sufficient to provide expression profiling of primary and metastatic tumors enabling network analyses to make way into routine clinical use to direct the design and adaptation of individualized treatment plans for patients over the course of their disease. In this future setting, synergistic modulation of multiple biological pathways, not necessarily limited to the primary target signaling pathway, will become the norm. Given the success of immune checkpoint inhibitors in melanoma and lung cancer, the combination of molecularly targeted therapies and immunotherapy/immunomodulatory therapies will be evaluated in the setting of HER2/neu overexpression. In the short term, the next 5 years, the robust pipeline of small-molecule

inhibitors will make its way forward; defining the clinical utility of individual agents and providing a larger armamentarium for the practicing clinician.

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### Executive summary

**History & biology of HER2/neu**
- Member of EGF receptor (EGFR) family.
- Complex heterodimer and ligand signaling network.
- Involved in normal development of cardiovascular and neurologic systems.

**HER2/neu-targeted therapeutics**
- Antibody-based therapies are the most advanced and broadly employed:
  - Trastuzumab
  - Pertuzumab
  - Trastuzumab emtansine (T-DM1)
- Small-molecule tyrosine kinase inhibitors have been approved and have a role in the treatment armamentarium:
  - Lapatinib
  - Afatinib
- Immunotherapeutic strategies
  - Tumor vaccines have advanced into clinical trials with suggestion of clinical benefit in some settings.
  - Adoptive cellular therapy and chimeric antigen receptor engineered T cells are in the pipeline.
- Modulators of HER2/neu expression and downstream signaling:
  - HSP90 inhibitors

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