

Novel therapeutic strategies targeting the epidermal growth factor receptor (EGFR) family and its downstream effectors in breast cancer

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From the early experience with tamoxifen to the current use of Herceptin[®], targeted therapy has been proven to be an important part of breast cancer (BC) treatment. In the last decade, advances in molecular biology have allowed scientists to design highly individualized, 'smart' pharmaceuticals, capable of manipulating the growth factor pathways and the genes that are involved in the development and maintenance of the malignant phenotype. The epidermal growth factor receptor (EGFR) family, as one of the best studied growth factor pathways in cancer, resembles a 'treasure island' by providing a wide range of biologically relevant targets involved in breast carcinogenesis. While a large number of new agents targeting this pathway are continually being tested in preclinical experiments, clinicians are witnessing the migration of some of these agents to daily practice. The aim of this review is to provide clinicians with an updated synopsis of the most advanced anti-erbB therapeutic strategies with activity against BC.

Key words: Akt, breast cancer, EGFR (ErbB) family, HER-2, MAPK

Targeted therapy for breast cancer: history and challenges

Our standards of care in medical oncology are largely based on empirical observations. More recently, advances in molecular biology have led to the identification of specific targets involved in fundamental steps of carcinogenesis. New biological targets have been extensively investigated as potential prognostic and/or predictive factors and a broad spectrum of promising biological agents are designed to block their critical function in development and maintenance of the malignant phenotype. There are already a few examples showing that, when a biologically relevant target is inhibited in some way, the patients whose tumors express that specific target can enjoy significant clinical benefit. This progress will undoubtedly represent a revolution in cancer therapy by leading to a shift in our clinical practice from non-specific use of anti-neoplastic agents to the use of specific, targeted ones. However, despite the enthusiasm generated in recent years, this is not a new concept in medical history, since 'targeted therapy' has long been known in breast cancer (BC) management, since the approval of anti-estrogen tamoxifen for the treatment of metastatic breast cancer (MBC) in the 1970s.

Targeted biological therapies have the advantage of maximizing efficacy while often reducing toxicity when compared with classical chemotherapeutic agents. Clinical development of these

agents requires different methodological procedures, mainly due to their different mechanism(s) of action. For example, they usually exert their desired effects at doses lower than their maximum tolerated dose, therefore the concept of 'optimal biological dose' is preferred to define their recommended clinical doses. In addition, verification of their biological effect at the target through appropriately selected surrogate pharmacodynamic markers constitutes an essential part of the trials investigating these agents. Biological agents target specific molecules, the expression or presence of which have to be determined in order to identify the specific patient group that is most likely to benefit from this therapy. One example is the determination of HER-2 status for trastuzumab (Herceptin[®]) therapy. Unfortunately, most of the tests developed for this purpose still need to be validated. This validation procedure might be time consuming, as exemplified by the measurement of estrogen and progesterone receptor, our traditional and best known predictive factors available for more than 30 years. Recent reports indicate that major inter-laboratory discrepancies still exist in measurement of hormone receptors in breast tumors [1]. The scenario regarding HER-2 is even more worrisome, since the benefit of trastuzumab has been proven to be limited to patients with HER-2 overexpressing/amplified tumors and treatment with this agent is associated with a non-negligible risk of cardiotoxicity. Poor inter- and even intra-laboratory concordance in HER-2 testing by immunohistochemistry (IHC) has long been reported. However, recent reports suggest that even fluorescence *in situ* hybridization is not 100% reliable [2]. This issue is of particular importance due to currently ongoing trials of trastuzumab in the adjuvant setting. Finally, it would be unrealistic to think that one could inhibit the contribution of a specific

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metabolic pathway to the carcinogenesis process by blocking one single critical molecule in the biological network, unless that molecule represents the sole mechanism for the development of a particular cancer, as in the example of STI-571 (Gleevec®) for chronic myeloid leukemia. The genomic instability of the tumor and the complex interplay between different signal transduction pathways (STPs) always provide several alternative ‘escape routes’ whenever the tumor’s survival is compromised. Therefore, we might need to evaluate the activity of several tightly interconnected biological pathways simultaneously in order to predict the desired effect of a biological agent. The recent literature investigating the role of phosphorylated forms of cell surface receptors and their downstream effectors provides valuable insights into our understanding of possible resistance mechanisms to targeted agents and suggests that it might also be important to analyze whether these receptors are in an activated state or not, in addition to checking their presence in the cell membrane [3].

A large body of biological agents targeting key molecules in the tumor cell machinery, interfering with metabolic pathways involved in cellular proliferation, angiogenesis, cell cycle regulation, apoptosis, etc., are in different phases of development. This review will focus mainly on the new agents targeting the members and downstream effectors of the epidermal growth factor receptor (EGFR) signaling pathway.

Materials and methods

A systematic literature search was performed to review the published clinical trials, reviews and abstracts focusing on the role of the EGFR signaling pathway in BC and the novel therapeutic strategies that target it. Three major sources were used: (i) the Medline search tool applying the words ‘breast cancer’ and the terms ‘EGFR’, ‘Her-2’, ‘Akt’, ‘MAPK’; (ii) a manual search of the proceedings books of the major international oncology meetings, including the American Society of Clinical Oncology (ASCO), American Association for Cancer Research (AACR), European Society of Medical Oncology (ESMO), European Cancer Conference (ECCO), San Antonio Breast Cancer Meeting (SABCM) and EORTC–NCI–AACR Molecular Targets and Cancer Therapeutics Symposium; and (iii) the reference lists of existing reviews and clinical studies.

Part I: the EGFR signaling pathway and its downstream effectors as promising targets

I-A. The EGFR signaling network

It is crucial to know each step in the complicated EGFR subway network in order to better understand both how this signaling network contributes to breast carcinogenesis and how the biological agents targeting this pathway function. The EGFR family of receptors consists of four closely related members: EGFR/ErbB1, HER-2/ErbB2, HER-3/ErbB3 and HER-4/ErbB4 [4]. All receptors have an extracellular domain (ECD) responsible for ligand binding, a helical transmembrane segment and an intracellular protein tyrosine kinase (TK) domain. At present, ten ligands have been identified to bind HER-1, HER-3 and HER-4: epidermal growth factor (EGF), transforming growth factor alpha (TGF- α), amphiregulin, heparin-binding epidermal growth factor (HB-EGF),

betacellulin, epiregulin and neuregulins 1 to 4 [5]. HER-2 is considered to be an ‘orphan’ receptor, since no specific direct ligand has been identified as yet, and increasing evidence suggests that it acts mainly as a co-receptor, increasing the affinity of ligand binding to the dimeric receptor complex. TK activity, which is required for receptor mediated cellular signaling, is present in all receptors except HER-3. Instead, HER-3 has six intracellular binding sites for phosphatidylinositol 3-kinase (PI3K), which is therefore a potent activator of this enzyme. The binding of ligands induces dimerization of two identical (homodimer) or different (heterodimer) receptors. The dimerization partner has an important impact on the type and number of downstream effectors activated and also on the downregulation mechanism of the ligand bound receptors. Signaling through HER-2 and -3 requires heterodimerization since, as stated, HER-2 has no known ligand and HER-3 lacks TK activity. Importantly, HER-2 is the preferred dimerization and signaling partner for all other HER receptors [6]. Upon dimerization, intracellular TK domains are phosphorylated, which in turn provide docking sites for several adaptor proteins and signaling enzymes. These proteins link upstream membrane receptor kinases to downstream intracellular protein kinases, which control a wide variety of cellular processes, including cellular proliferation, apoptosis and angiogenesis. Not surprisingly, the involvement of multiple ligands, paired combinations of four receptors and plenty of intracellular downstream effectors (signal processing units) result in extensive diversification of EGFR signals mediated by the EGFR family of receptors [7].

PI3K and mitogen-activated protein kinase (MAPK) pathways are the two major downstream signaling cascades activated by the EGFR family of receptor TKs (Figure 1). The intracellular part of the receptor determines the type and intensity of the downstream signaling pathway that is activated. For example, EGFR (ErbB-1), in contrast to other members of the HER family of receptors, induces relatively weak activation of the PI3K pathway since this receptor has no intracellular binding sites for PI3K. Molecules targeted to the downstream effectors of the EGFR family of TKs are in various stages of development [8–10] and will be briefly discussed in the second part of this review.

(1) Ras–Raf–MAPK pathway. Ras proteins are small guanine nucleotide binding proteins serving as a key intermediary in several STPs, thereby controlling a wide variety of cellular processes including growth, differentiation, apoptosis and cytoskeletal organization [11]. Ras proteins are activated in response to a wide variety of intracellular and extracellular stimuli. Activated Ras is rapidly converted to its guanosine diphosphate (GDP)-bound inactive state by hydrolysis of the bound guanosine triphosphate (GTP) by an intrinsic GTPase activity. This mechanism of inactivation is thought to be impaired in mutant Ras, which remains in the activated form and conveys uncontrolled proliferative signals continuously, despite the absence of stimulation. Ras proteins are mutationally activated in ~30% of human cancers, including BC [11].

Ras is synthesized as an inactive cytosolic propeptide and is localized to the inner surface of the plasma membrane only after a series of post-translational modifications, facilitating its association with plasma membrane. Protein farnesyl transferase, which

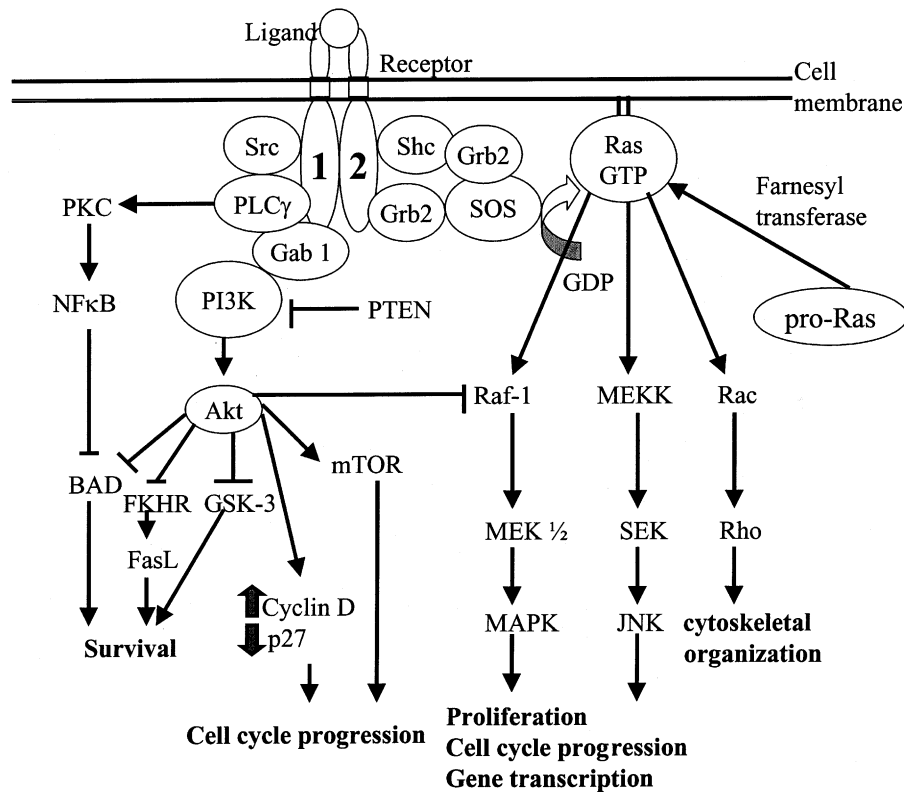


Figure 1. Downstream effectors of epidermal growth factor receptor (EGFR) signaling pathway. PLC γ , phospholipase C; PKC, protein kinase C; NF κ B, nuclear factor κ B; PI3K, phosphatidylinositol 3-kinase; MEK, mitogen-activated protein kinase; MEKK, mitogen-activated protein kinase kinase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin.

catalyses the most important step in activation of Ras, represents a novel target for the development of new anticancer agents.

Ras can be activated by receptor TKs (Figure 1). Tyrosine phosphorylated receptors provide binding sites for the adaptor protein Grb2 (growth factor receptor-bound protein 2), which recruits Ras activator protein SOS (son-of-sevenless guanine nucleotide exchange factor). Upon binding to Grb2, SOS mediates the activation of Ras by facilitating its switch from the inactive GDP-bound form to the GTP-bound active form. Then, GTP-bound Ras can activate several downstream effector pathways. Raf-1, another protein kinase, is one of these effector molecules. It phosphorylates MEK1 (MAPK/Erk kinase) and MEK2, which in turn phosphorylate the MAPKs, ERK1 and -2 (extracellular signal-regulated kinases). Once activated, Erk1 and -2 translocate into the nucleus, where they phosphorylate a variety of substrates, including nuclear transcription factors, and ultimately lead to transcription of target genes associated with cellular proliferation. Other substrates for Ras signaling include Rac/Rho (small GTP-binding proteins), PI3K and mitogen-activated protein kinase kinase (MEKK). The Rac-rho pathway is involved in cytoskeletal organization, the PI3K pathway is associated with cell survival, and the MEKK and MAPK pathways mediate cellular proliferation signals. The MAPK pathway is activated preferentially by mitogens, growth factors and tumor promoters, and the other downstream pathways mediated by Ras signaling are stimu-

lated by inflammatory cytokines, hormones and various forms of stress stimuli. MEKK is a serine threonine phosphatase, which activates another MAPK family member JNK (Jun N-terminal kinase) via SEK (stress-activated protein kinase), and the activation of this pathway is associated with apoptosis and/or proliferation depending on the dynamic balance between different STPs. JNK activates nuclear transcription factor c-Jun, a major component of activator protein-1 (AP-1). The other component of AP-1, c-fos, is activated by the MAPK pathway mediated through EGFR signaling [12]. The generation of AP-1 is thought to play an important role in the development of endocrine resistance in BC as one of the critical intersection points between estrogen receptor (ER) and EGFR signaling pathways [13]. Many STPs impinge on Ras-mediated signaling at different levels, so that the net effect of a given stimulus reflects merely the balance of crosstalk among these pathways.

(2) *PI3K-Akt pathway.* The PI3K family of enzymes has been linked to many aspects of malignant transformation, including increased proliferation, growth, motility, invasiveness, metastases, angiogenesis and cell survival. Since the PI3K family of kinases, their targets and regulators are implemented in a wide variety of human tumors, they are considered to be potential new targets. PI3K is activated by a diverse set of stimuli including cell surface receptors (G-protein-coupled receptors, receptors with TK activity), tyrosine phosphorylated proteins (e.g. insulin-regulated

substrate, IRS) and small G proteins (e.g. Ras). EGFR (ErbB-1), in contrast to other members of the HER family of receptors, induces activation of the PI3K pathway relatively weakly, since this receptor has no intracellular binding sites for PI3K. Upon activation, PI3K phosphorylates the inositol ring of membrane phosphatidylinositols (PIP3 and PIP2), which recruit several proteins to the cell membrane such as PKB (protein kinase B, also known as Akt), PDK1 (3-phosphoinositide-dependent protein kinase) and PDK2, small proteins of the Ras family, enabling these signaling proteins to come into close contact with their substrates [14]. PKB/Akt, a serine threonine kinase, is thought to be the major player in the PI3K pathway; however, it can be activated by other molecules independent of the PI3K pathway, such as PKA (protein kinase A), the calmodulin-dependent kinase pathway, and cellular stresses like heat shock and hyperosmolarity [15]. Among three Akt proteins, Akt2 and Akt3 were shown to be activated in human BC samples [14]. The binding of PKB to phosphatidylinositols is followed by its phosphorylation and activation by PDKs. Once activated, Akt regulates a number of proteins involved in the regulation of the cell cycle (cyclin D and E2F), apoptosis and cell survival [p21, p27, nuclear factor κ B (NF- κ B), Forkhead proteins, caspases and BAD] [16]. Akt also phosphorylates a number of other proteins, the functions of which are closely related to BC, such as BRCA1 and ER [17, 18]. It has been shown that ER- α can bind PI3K and activate PI3K/Akt pathway in an estrogen-independent manner [18–20].

Many other STPs converge on the PI3K pathway at the level of Akt. For example, Ras activates the PI3K pathway, while Akt also regulates the activity of the Ras–Raf–MAPK pathway through phosphorylation and inactivation of Raf [21]. PI3K signaling can be inhibited by protein phosphatases, the prototype of which is the tumor suppressor gene product PTEN (phosphatase and tensin homolog deleted on chromosome 10) [22]. Loss of PTEN activity due to either mutations or gene deletions is associated with malignancies such as Cowden’s syndrome. mTOR (mammalian target of rapamycin) kinases are among the downstream targets of Akt, and they are thought to link mitogenic stimulation to protein synthesis and cell cycle regulation [23]. The inhibition of mTOR blocks the progression of the cell cycle at the G₁ phase. Tumor cells without PTEN activity are considered to be more sensitive to mTOR inhibitors due to their increased level of Akt activity [24].

I-B. The EGFR signaling pathway in BC

The EGFR family of receptors is involved in the regulation of normal breast development [25, 26]. Preclinical data suggest that all compartments of EGFR signaling network including ligands, receptors and downstream effectors are implicated in the development and progression of BC [16, 27]. Overexpression of HER-2 in transgenic mouse mammary glands has been shown to promote oncogenic transformation and development of malignant phenotype [28–31]. In line with this data, HER-2 activation has been reported to increase metastatic/invasive potential and to induce progression of cell cycle by disrupting the delicate balance between cyclins and the endogenous CDK inhibitors in BC cell lines [32, 33]. Hence, not surprisingly, HER-2 overexpression is associated with aggressive disease biology and reduced survival in BC

patients [34, 35]. These preclinical findings are further supported clinically by the success of trastuzumab, a monoclonal antibody against HER-2, which led to improved survival, longer time to progression, higher response rate and longer duration of response in MBC patients with HER-2 overexpressing tumors [36].

Numerous studies have also reported that EGFR (HER-1) overexpression is a poor prognostic factor in BC, often associated with advanced disease [37–41]. While HER-2 overexpression is shown to be an early event in BC development, HER-1 seems to be involved in later stages [42]. The expression of both receptors is inversely correlated with the ER status of the tumor and HER-1/2 heterodimers have been shown to increase metastatic potential of BC cell lines [43, 44]. Accumulating data suggest that EGFR and HER-2 also predict for a poor response to endocrine therapy, and that the mutual interaction between ER and growth factor pathways seems to hotwire the events inducing endocrine resistance in ER-positive breast tumors [45–52]. Acquisition of resistance to endocrine therapy was shown to be associated with upregulation of EGFR signaling in BC cell lines [53, 54]. ErbB receptors enhance ER signaling either by directly activating ER or through activation of MAPK and Akt [55–57]. Moreover, there is pre-clinical evidence that estrogen itself suppresses the transcription and expression of ErbB receptors [58]. So far, preclinical data have shown that agents blocking HER-1 or HER-2 driven signaling pathways can restore hormone sensitivity in endocrine-resistant, HER-2 overexpressing breast tumors, delay the development of endocrine resistance and enhance the antiproliferative effect of endocrine agents [59–66]. These effects are partly mediated by the inhibition of downstream signaling cascades involved in the development of endocrine resistance. The expression of MAPK or Akt has been reported to predict a worse clinical outcome in patients treated with endocrine therapy [67, 68]. Both MAPK and Akt are capable of activating ER in a ligand independent manner by phosphorylating ER at Ser-118 and -167, and activated MAPK can also enhance ER signaling by recruiting nuclear receptor co-activators [56, 57, 69, 70]. The impact of EGFR downstream effectors on cell cycle progression may also indirectly mediate endocrine resistance. For example, Akt and MAPK phosphorylate the cell cycle inhibitor p27, which is required for the G₁ arrest mediated by anti-estrogens [71]. In addition, phosphorylation by MAPK leads to destabilization and proteasome-mediated degradation of p27 in BC cell lines, therefore disturbing its inhibitory effect on cell cycle progression [72]. Importantly, Akt-induced cytoplasmic sequestration of p27 in human BC specimens has recently been shown to be associated with reduced patient survival [73–75]. Endogenous Akt, either constitutively activated via PTEN mutations and overexpression of ErbB receptors or induced by therapeutic agents, has been shown to promote BC cell survival [76]. Induction of Akt activity in response to commonly used therapeutic modalities in BC is considered to be a potential mechanism of therapeutic resistance. Indeed, inhibition of Akt by a targeted agent has been shown to potentiate trastuzumab-, doxorubicin-, paclitaxel- and tamoxifen-mediated apoptosis in BC cell lines [76].

EGFR signaling also seems to be vital for the survival of ER-negative BC. Akt-3 is overexpressed in ER-negative breast

cell lines and in tissue samples taken from patients [77]. In addition, EGF-induced activation of PKC (one of the downstream substrates of PI3K) and NF- κ B has been shown to mediate cellular proliferation in ER-negative BC cells [78].

Regarding the other receptors of the EGFR family, HER-3, the kinase deficient member, has also been shown to be expressed in BC, while the expression of HER-4 is uncommon in breast carcinomas compared with normal breast epithelium and is associated with favorable prognostic factors [79–81]. Of note, HER-2/HER-3 heterodimers have the highest mitogenic potential and are constitutively activated in BC cells with HER-2 gene amplification [4, 82].

Consequently, all these data render the EGFR family of receptors, their ligands and their downstream effectors rational targets for the development of novel antitumor strategies in BC.

Part II: novel strategies targeting the EGFR family and its downstream effectors

I. Targeting HER-2 signaling

(a) *Monoclonal antibodies.* Herceptin[®] for the treatment of BC and Gleevec[®] (imatinib mesylate, STI-571) for the treatment of chronic myeloid leukemia have provided the most prominent initial proof that targeted cancer therapy can translate into improved clinical outcomes. Trastuzumab represents the only biological targeted therapy for BC in routine clinical practice today. The establishment of the unique role of HER-2 in malignant transformation in preclinical models, coupled with the biological significance of HER-2 overexpression in BC and the preclinical demonstration of the antitumor activity of monoclonal antibodies (mAbs) directed against HER-2, encouraged the development of this drug [83–85]. HER-2 is overexpressed in 20–25% of breast tumors and is associated with a poor prognosis [83]. Trastuzumab is a humanized antibody, composed of an antigen-binding component (from the murine monoclonal antibody 4D5) combined with human immunoglobulin G, and is shown to have a single agent activity of 15% in pre-treated patients and 26% in previously untreated patients [86, 87]. Anti-HER-2 monoclonal antibodies affect tumor growth both directly by altering the receptor's signaling properties and indirectly by antibody-dependent cell-mediated toxicity and complement-dependent cytotoxicity. Although trastuzumab's direct inhibitory actions on breast tumor growth and proliferation are not precisely characterized, it is thought to act by: (i) downregulating Her-2/neu receptor; (ii) blocking receptor dimerisation; (iii) altering Her-2 phosphorylation state and/or signaling properties; (iv) altering gene expression; (v) inducing apoptosis; and (vi) inhibiting angiogenesis [88–90]. The success obtained in MBC clinical trials is only the beginning of the trastuzumab story in BC; the best way to measure HER-2, the best schedule and duration of trastuzumab therapy, the best combinations with other agents and the role of trastuzumab in early BC are among the key questions that remain to be answered. Interestingly, only ~40% of patients with HER-2 overexpression respond to trastuzumab. Recent investigations focused on the role of the co-expression patterns of Her dimers, the proportion of activated

(phosphorylated) receptors and the impact of several other pathways, e.g. IGF-IR (insulin-like growth factor receptor I) on HER-2-mediated signal transduction, in order to unravel the mechanisms of therapeutic resistance to trastuzumab [3, 88, 91]. Preclinical data, demonstrating that the HER-2 signaling pathway is highly interactive with other biological pathways such as ER signaling, and that trastuzumab has synergic or additive activity with multiple cytotoxic agents, led to a number of phase I/II trials being conducted to evaluate the efficacy of this agent in combination with endocrine agents (e.g. tamoxifen, aromatase inhibitors), other biological agents (e.g. ZD1839, OSI 774, celecoxib, flavopiridol, R115777), cytokines (interleukin-2 and -12) and cytotoxic agents (e.g. vinorelbine, carboplatin) in MBC patients [92].

Intensive research is also ongoing in order to determine the mechanism of trastuzumab-mediated cardiotoxicity, the most relevant side effect of trastuzumab. A recent *in vivo* trial has shed some light on this issue by studying the role of ErbB2 in ErbB2 conditional mutant mice that have ventricular-restricted deletion of ErbB2. These mice, unlike ErbB knockout mice, were viable without morphological cardiac defects at birth. They were shown to develop dilated cardiomyopathy and the cardiomyocytes isolated from these mice were more sensitive to anthracycline toxicity [93].

A few new agents designed to increase the efficacy of trastuzumab are currently being tested in preclinical trials. One of them, Herceptin-DM-1, represents a combination of trastuzumab with a highly potent microtubule toxin, mytansinoid. It has been shown that the immunoconjugate has more potent antitumor activity compared with trastuzumab alone in both HER-2-responsive and -resistant breast tumor models [94]. The cytotoxicity of the DM-1, which is released upon internalization of the conjugate, is confined only to HER-2-expressing cells [95]. Two other humanized mAbs targeting HER-2 are being developed: 2C4 and MDX-H210. 2C4 is directed to an epitope of ECD of HER 2, involved in the dimerization process, and does not require high HER-2 expression for antitumor activity. It inhibits MAPK and PI3K pathways, prevents Her-2/Her-3 heterodimerization and sensitizes tumor cells to chemotherapy in preclinical experiments [96, 97]. MDX-H210 is a bispecific mAb directed against both HER-2 and the Fc gamma receptor, which mediates phagocytosis and cytolysis of HER-2 overexpressing BC cells by macrophages [98]. Granulocyte colony stimulating factor (G-CSF) and interferon γ were found to enhance the immune-based cytotoxic potential of MDX-H210 and therefore were co-administered with MDX-H210 in several phase I trials [99, 100]. Available data from phase I studies have shown that MDX-H210 is biologically active and able to induce temporary disease stabilization in patients with HER-2-overexpressing advanced tumors [101].

(b) *Tyrosine kinase inhibitors.* Another mechanism by which one can target HER-2 is the inhibition of its TK activity. The safety, tolerability and pharmacokinetics of a new selective HER-2 tyrosine kinase inhibitor (TKI), TAK-165, is currently being evaluated in a multicenter phase I trial in HER-2 expressing MBC patients [102, 103]. Curcumin and emodin, two natural compounds derived from plants, were also shown to possess anti-TK activity in preclinical experiments [104, 105]. Emodin inhibits HER-2 TK

activity, suppresses the transformation of HER-2 overexpressing BC and sensitizes them to chemotherapeutic agents, including cisplatin, doxorubicin, etoposide and paclitaxel [104, 106]. Curcumin inhibits HER-2 TK and depletes HER-2/neu protein in HER-2-expressing BC cell lines [105]. Compound 820, an irreversible HER-2 TKI, and CP 654577, a selective HER-2 TKI, are the two other new agents with potent activity against HER-2-expressing tumor cell lines, *in vitro* and *in vivo* [107, 108].

(c) *Antisense strategies.* The growth of HER-2-overexpressing cell lines is shown to be inhibited by a plasmid that expresses HER-2/neu antisense RNA. This method leads to decreased gene expression at both translational and transcriptional levels. Moreover, regarding the growth inhibition of BC cells, the antisense HER-2/neu RNA is synergic with trastuzumab and a number of cytotoxic agents [109].

(d) *Active immunization against HER-2 by vaccines.* The generation of active immune response to HER-2 represents an attractive alternative to passive immunity provided by mAbs. The major goal of cancer vaccines is to induce a specific immune response to a tumor-specific antigen and establish a long-term immunological memory for this reaction. Several vaccination strategies using either whole HER-2 or its components such as ECD, synthetic peptides and naked DNA are being evaluated. A phase I/II clinical trial is currently assessing the safety and the tolerability of AutoVac[®] HER-2 DNA pharmaccine in Europe. This vaccine stimulates the patient's own immune system to induce a strong killer cell (CTL) response against HER-2-overexpressing tumor cells [110]. Another vaccine, called AutoVac[®] HER-2 protein pharmaccine, has been shown to be at least as effective as trastuzumab in controlling the growth of breast tumors in xenograft models [110]. Disis et al. [111, 112] reported that the development and maintenance of T-cell immunity to HER-2 protein after active immunization with HER-2 peptide vaccines is possible in patients with HER-2-overexpressing breast, ovarian and non-small-cell lung cancer (NSCLC), while keeping an acceptable toxicity profile. HER-2 protein-based vaccination strategies containing ECD fused with portions of the intracellular domain (ICD) of HER-2 are thought to be more immunogenic and have been shown to elicit antibody response to HER-2 in animal models. Furthermore, several adjuvants are being combined with HER-2/neu antigens in order to generate a substantial immune response in the host. A phase I HER-2 vaccine trial with fused HER-2 ECD and ICD combined with an adjuvant is currently being planned in order to evaluate the immunogenicity and safety of this vaccine in high-risk BC patients. Tumor derived heat-shock proteins (HSPs) have been shown to evoke an immune response in preclinical experiments [113]. Therefore, it is thought that the combination of an HSP with a tumor-specific antigen may enhance the immune response against that particular antigen. A recombinant HSP110-HER-2 vaccine (HSP110 is non-covalently complexed to the ICD of HER-2 during heat shock *in vitro*) has been shown to elicit both cellular and humoral immune response against the ICD [114]. Such an approach is not patient specific, is applicable to all tumors expressing HER-2 and does not require tumor specimens to prepare the vaccine.

(e) *Gene therapy.* Transcriptional regulators, such as adenoviral 5E1A (adenovirus type 5 early region 1A) gene product, have been shown to downregulate the HER-2 promoter, repress the HER-2 gene and reverse the transformed phenotype in preclinical studies [115]. A phase I gene therapy clinical trial, which was conducted on the basis of these promising preclinical data, has already been completed in advanced breast and ovarian cancer patients [116].

(f) *Other strategies.* (i) Targeted immune effector cells. The antitumoral activity of immune effector cells can be modified by viral transduction methods. It has recently been reported that genetically modified natural killer (NK) cells expressing a chimeric antigen specific for tumor-associated ErbB-2 selectively, and efficiently lysed ErbB-2-expressing breast tumor cells. Such promising experimental data might allow NK-based therapeutic strategies for ErbB-expressing tumors [117].

(ii) HSP inhibitors. Heat shock proteins have emerged as attractive targets for new anticancer drugs because these molecules modulate the STPs controlling tumor cell growth and survival. These molecules are not mutated in cancer, but they facilitate malignant transformation by enhancing the activity of many oncogenic growth factor receptors, kinases and transcription factors. HSP90 is required for the refolding of proteins in response to environmental stress and the conformational maturation of several signaling proteins [113]. The function of HSPs can be inhibited by compounds that bind to a conserved pocket in the amino terminal of the protein. One such compound is 17-AAG (17-allylamino-17-demethoxygeldanamycin), which is currently in phase I clinical trials in advanced cancer patients [118]. Agents targeting HSP90 chaperone stimulate the dissociation of the client proteins from the chaperone complex and cause their ubiquitin-dependent degradation by the proteasome pathway. HER-2 TK is one of the most sensitive targets for HSP90 inhibitors. HER-2-overexpressing cell lines are found to be particularly sensitive to the antiproliferative effects of 17-AAG. Treatment with 17-AAG results in the inhibition of Akt activity in BC cell lines through the abrogation of HER-2/HER-3 heterodimers (due to degradation of HER-2 and dephosphorylation of HER-3 and uncoupling of PI3K from HER-3 [119, 120]. Interestingly, the growth arrest and apoptosis induced by 17-AAG is shown to be dependent not only on the overexpression of HER-2, but also on the co-expression of HER-3 and the activation of the PI3K pathway.

(iii) Histone deacetylase inhibitors. The inhibitors of histone deacetylators are another group of new promising agents that lead to the acetylation of histone proteins and alter chromatin structure. They have been shown to deplete the levels of several proteins (e.g. HER-2, EGFR, Raf-1) that are normally stabilized by binding to HSP90 in cells. These agents are in preclinical stages of development [121].

(iv) Proteasome inhibitors. The proteasome is an enzyme complex, present in all cells, responsible for the proteolytic degradation of cellular proteins that are involved in inflammation, cell cycle regulation, gene expression, cellular growth and differentiation [122]. If the function of the proteasome is impaired, unregulated degradation of vital cellular proteins, such as cell cycle regulatory proteins or growth factor receptors (ErbB family), can

lead to uncontrolled proliferation. The proteasome pathway has been implicated in HER-2 protein degradation [72]. It is possible that proteasome inhibition can increase the number of Her-2 receptors, hence increasing the efficacy of trastuzumab therapy. Accordingly, a phase I dose escalating study of PS-341, a proteasome inhibitor, in combination with trastuzumab in HER-2-over-expressing MBC patients is ongoing at our institution.

II. Targeting HER1/ErbB1 (EGFR)

Deregulation of EGFR (ErbB1), another member of the EGFR family, has also been implicated in the development of a number of human tumors including BC [123]. The mutated receptor (EGFR vIII), which is constitutively active in the absence of ligands, and the overexpression of EGFR represent the two most important mechanisms causing EGFR deregulation [123, 124]. Dysfunctional receptor downregulation and the overexpression of ligands are other mechanisms leading to increased signaling through EGFR [123]. Of note, EGFR itself is a critical downstream element of other signaling systems induced by integrins and cytokine receptors. Ultimately, all these factors lead to high TK activity, which facilitates signal transduction to the nucleus, promoting reduced apoptotic potential and cell cycle progression. Currently, overexpression is the only mechanism by which EGFR deregulation can be detected (by IHC). In contrast to HER-2 overexpression, which is generally due to gene amplification and predicts response to trastuzumab therapy, the overexpression is only one of the several mechanisms by which EGFR deregulation occurs, and data regarding the correlation between EGFR overexpression and response to EGFR targeting agents are conflicting. The EGFR expression rate in BC is in the range of 14–91%, depending on the method of assessment, and is almost always caused by increased receptor synthesis [125]. EGFR mutations have been reported in 78% of BC cases by RT-PCR and 27% by IHC [126, 127]. Ongoing trials with anti-EGFR treatment strategies in BC will define the prognostic significance of EGFR expression and its potential to predict response.

There are a number of ways of targeting EGFR deregulation [7, 123] (Table 1), some of which will be described below.

(a) *Ligand-based strategies.* Toxin conjugated ligands are generated by conjugating a potent cellular toxin with one of the ligands of the HER family receptor. For example, the TGF- α -pseudomonas exotoxin A conjugate has been evaluated in a phase I trial [123]. The generation of systemic anti-ligand immunity in the host, either by active or passive immunization with ligands, can reduce the ligand concentration for receptor activation. Additionally, ligand/anti-EGFR antibody complexes, such as TGF- α single chain anti-EGFR, can also interfere with EGFR signaling at the receptor level [128].

(b) *Monoclonal antibodies.* Given the success with trastuzumab therapy and the contribution of EGFR signaling in breast carcinogenesis, the potential role of other anti-EGFR mAbs in BC whet the oncologist's appetite for new therapeutic successes. So far, the antitumor effect of several other anti-EGFR mAbs has only been tested in BC cell lines.

Blocking antibodies act as receptor antagonists and prevent ligand binding. One major limitation of antibody treatments is the

development of anaphylactic reactions due to potential immunogenicity. IMC-C225 (Erbbitux[®]), a chimeric monoclonal antibody directed against EGFR, is in the late stages of clinical development (phase II–III) for colorectal, pancreatic, and head and neck cancer [129–131]. Among other anti-EGFR mAbs, there are three fully humanized mAbs in clinical evaluation: ABX-EGF is currently undergoing phase I/II evaluation, h-R3 has completed phase I evaluation [132, 133] and EMD 72000 has shown promising activity with acceptable toxicity as a single agent in a phase I trial [134]. The combined treatment with h-R3 and radiotherapy induced objective responses in advanced head and neck cancer patients in a phase II trial [135]. Two other anti-EGFR mAbs, ICR-62, a rat anti-EGFR mAb, and EMD 55900, a murine anti-EGFR mAb, inhibit the proliferation of head and neck tumor cell lines and have been evaluated in phase I trials [130, 136]. However, in a phase II trial of glioblastoma patients EMD 55900 did not demonstrate favorable antitumor activity [137]. mAb 806, which is currently being evaluated in preclinical experiments, specifically recognizes amplified and mutant versions of the EGFR that are expressed by different types of tumors, including gliomas [138].

The generation of an antitumor immune response with concurrent inhibition of EGFR can be achieved by bispecific anti-EGFR mAbs that can target both the EGFR and the immune effector cells. In this group of agents, MDX-447 is currently being investigated in phase I/II trials [139].

Toxin-conjugated antibodies represent another modality of extracellular targeting. Usually, a potent cellular toxin such as pseudomonas toxin A or diphtheria toxin is conjugated to an anti-EGFR antibody to generate an immunotoxin conjugate. These conjugates enter the cell via receptor-mediated endocytosis and kill the target cell ultimately through inhibition of protein synthesis. Although the exact relationship between tumor EGFR expression and response has not yet been established, interesting treatment strategies are being investigated in order to enhance the antitumor efficacy of anti-EGFR mAbs, particularly for those tumors with low levels of EGFR expression. For example, the combination of TNF- α , which increases EGFR expression, with anti-EGFR mAbs (EMD 55900 or EMD 72000) has been shown to improve antitumor activity of anti-EGFR mAbs in animal models [140].

Many anti-EGFR strategies could be adapted directly to EGFR vIII (mutated EGFR), which has the advantage of being specifically expressed only on the cancer cells [141]. Therapeutic mAbs against mutant EGFR may find extensive clinical applications in those tumors that frequently express this variant, such as gliomas and non-small-cell lung carcinomas. The optimal methodology for EGFR vIII status determination and the confirmation of its reported high rate expression in breast tumor specimens need to be achieved in parallel with the ongoing experimental and clinical trials testing anti-EGFR vIII strategies.

(c) *Tyrosine kinase inhibitors.* TKIs are small molecules that bind to the ATP binding site in the intracellular domain of receptors and block TK activity either reversibly or irreversibly. One major advantage, compared with mAbs, is their ability to block EGFR with mutated or deleted ECD. In addition to selective EGFR TKIs

Table 1. Agents targeting EGFR pathway (in preclinical and clinical phases of development)

Target	Agent	Mechanism of action	Phase of development	Surrogate markers evaluated	Adverse effects
EGFR	IMC-C225 (Erbix [®]) chimeric	mAb	Phase III – head and neck cancer; phase II – colon and pancreatic cancer; preclinical – combination with cytotoxic agents in BC cell lines	pMAPK (Erk1/2), pAkt, Ki67 in skin	Acneiform rash, fever, chills, asthenia, nausea
	ABX-EGF (humanized mAb)		Phase I/II – renal, prostate, NSCLC and esophageal cancer	NA	Acneiform rash
	h-R3 (humanized mAb)		Phase II – head and neck cancer	NA	Hypotension, fever, tremor, nausea, somnolence
	EMD 55700 (rat mAb)		Phase II – GM (unfavorable activity)	NA	Few side-effects: rash, elevation in transaminases
	EMD 72000 (humanized mAb)		Phase II – ovarian and head and neck cancers	NA	Rash (DLT: headache and fever)
	ICR 62 (murine mAb)		Phase I	NA	Fever, rigor, chest pain
Mutant EGFR	MAb 806	mAb against EGFR vIII and EGFR-over- expressing cells	Preclinical	NA	NA
	Y10	mAb against human and murine mutated EGFR	Preclinical	NA	NA
EGFR and immune effector cells	M26.1 F(ab') ₂ -CD 3 (lymphocyte)	Bispecific anti- EGFR mAb	Preclinical	NA	NA
	MDX-447-CD64 (IgGR)		Phase II – head and neck cancer (ongoing)	NA	Headache, fever, chills, hypertension, myalgias, nausea edema, fatigue, arrhythmia (DLT in phase I: hypotension)
	H22-EGF-CD64 EGFR/CD86		Preclinical Preclinical	NA NA	NA NA
EGFR	ZD1839 (Iressa [®]) p.o.	Reversible TKI	Phase III – NSCLC; phase II – breast, head and neck and renal cancers; phase I/II – colon and prostate cancers	pEGFR, pMAPK, pAkt, p27 ^{KIP1} , Ki 67, c-fos in skin	Acneiform rash, diarrhea, nausea, transient elevation in transaminases
	OSI 774 (Tarceva [®]) p.o. (CP 358,774)		Phase III – NSCLC and pancreatic cancer; phase II – head and neck, ovarian and BCs	pEGFR, pERK, pAkt in skin; changes in 18-FDG by PET scan	Rash, diarrhea, nausea, fatigue, headache, transient elevation in transaminases
	PD 153035		Preclinical	NA	NA
EGFR, HER-2	PKI 166 p.o.		Phase I completed	pEGFR, pMAPK, pAkt in skin and hair follicles	Nausea, vomiting, diarrhea, rash, fatigue, reversible transaminase elevation
	GW 2016 p.o.		Phase I PK study – healthy volunteers; phase I trials underway	NA	Rash, diarrhea, headache, elevation of transaminases

Table 1. (Continued)

Target	Agent	Mechanism of action	Phase of development	Surrogate markers evaluated	Adverse effects
EGFR, HER-2, HER-4	CI 1033 p.o.	Irreversible TKI	Phase I completed; phase II – BC	pEGFR, pHER-2, p27, Ki67 in skin and tumor tissue	Nausea, vomiting asthenia, diarrhea (DLT: stomatitis, rash, reversible hypersensitivity)
	PD 158780		Preclinical	NA	NA
	BIBX1382BS		Preclinical	NA	NA
EGFR	AG 1478	TKI	Preclinical	NA	NA
	PD 169414		Preclinical in BC cell lines	NA	NA
	PD168393		Preclinical	NA	NA
	EKB 569		Preclinical	NA	NA
	LFM-A12		Preclinical in BC cell lines	NA	NA
	WHI-P97		Preclinical in BC cell lines	NA	NA
EGFR	scFv (225)	Ligand + anti-EGFR antibody complexes	Preclinical	NA	NA
	scFv (14 ^{E1})		Preclinical	NA	NA
EGFR ligand	EGF–genistein	Toxin-conjugated ligand	Preclinical in BC cell lines	NA	NA
	EGF–diphthera toxin		Preclinical	NA	NA
	EGF–Rnase		Preclinical in BC cell lines	NA	NA
	EGF–dextran		Preclinical	NA	NA
	TGF- α –ricin A		Preclinical	NA	NA
	TGF- α –ETA		Phase I	NA	–
EGFR RNA	Antisense oligonucleotides	Antisense molecules	Preclinical in BC cell lines	NA	NA
	Ribozymes		Preclinical	NA	NA
EGFR	FD 137	TKI + cytotoxic agent complexes	Preclinical	NA	NA
	BJ 2000		Preclinical	NA	NA
	SMA 41		Preclinical	NA	NA
Active immunization against EGF	EGF-P64K	Recombinant EGF vaccine	Phase II – advanced solid tumors	NA	Local itching, erythema at the site of injection

EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; NSCLC, non-small-cell lung cancer; GM, glioblastoma multiforme; DLT, dose-limiting toxicity; NA, not available; PK, pharmacokinetics; scFv, single chain antibody fragment; FDG, fluorodeoxyglucose; PET, positron emission tomography; p.o., orally; BC, breast cancer; ETA, pseudomonas toxin A; mAb, monoclonal antibody; TKI, tyrosine kinase inhibitor; MAPK, mitogen-activated protein kinase.

(e.g. ZD1839, OSI-774), some of these agents are capable of inhibiting both HER-1 and HER-2 TKs, so called 'dual inhibitors', while others can inhibit the TK activity of HER-1, HER-2 and HER-4 and are known as 'pan-erbB inhibitors'.

ZD1839 (Iressa[®]) and OSI-774 (Tarceva[®]) are the two TKIs in more advanced stages (phase III) of clinical development, particularly in NSCLC. They are also the two TKIs that are in the most advanced stage (phase II trials) of development in MBC. An open-label multicenter phase II study evaluated the efficacy and safety of ZD1839 in 63 heavily pre-treated advanced breast cancer (ABC) patients who had visceral-dominant disease [142]. The reported clinical benefit rate was 4.8%, with one partial response (PR) and two stable diseases (SD) lasting ≥ 6 months. The median progression-free survival and survival were 57 and 144 days, respectively. Overall, the treatment was well tolerated with a toxicity pattern similar to the one observed in single agent trials in advanced NSCLC. One-quarter of the patients experienced grade 3–4 toxicity, primarily grade 3 diarrhea, nausea and rash. Interestingly, a marked relief of bone pain was observed in 42% of patients. The interim results of another phase II study of ZD1839 (500 mg/day) in ABC patients with ER-negative and tamoxifen-resistant ER-positive tumors were recently presented [143]. Among the 22 patients enrolled to date, two had a PR and 10 patients had SD at 4 weeks; roughly one-third of patients experienced rash, most of which was grade 1 or 2 in severity. OSI-774 (150 mg/day) has also been evaluated in an open-label multicenter phase II study in ABC patients refractory to prior chemotherapy [144]. Two cohorts of patients were accrued: the first cohort ($n = 47$) included patients who received prior therapy with an anthracycline, a taxane and capecitabine, and the second ($n = 22$) group consisted of patients who had tumor progression during prior chemotherapy. Two PRs, one in each cohort, were reported and median time to progression was 43 days. More than 50% of patients experienced an acneiform rash but again, the severity was generally mild. Diarrhea, asthenia and nausea were the other treatment-related side-effects, and no correlation was detected between the number of responses and EGFR expression. Despite encouraging preclinical data, these somewhat disappointing initial efficacy results of both ZD1839 and OSI-774 underscore the need for a better understanding of the EGFR biology in BC. Additionally, it may also be argued that the study populations had advanced stage disease, which is fairly refractory to conventional treatments. Therefore, further evaluation of EGFR TKIs in different settings, such as less heavily pre-treated patients, and probably in combination with other drugs, is needed.

Accumulating data suggest that TKIs may also be useful in the treatment and chemoprevention of ductal carcinoma *in situ* (DCIS) [145]. Preclinically, ZD1839 has been shown to inhibit proliferation in DCIS even more effectively than trastuzumab [146, 147] and a phase II study is currently assessing its role in this setting. A recent *in vivo* study highlighted the potential of ZD1839 as a preventive strategy in BC [148]. ZD1839 has been shown to delay development of mammary tumors in transgenic mice that overexpress HER-2 in mammary tissue and usually develop ER-negative tumors over time.

Given the intensive interplay between estrogen receptors and the EGFR signaling pathway (see Part I-A above) and also the fact that anti-EGFR agents are also capable of enhancing the activity of several cytotoxic drugs, many other phase II trials are assessing the efficacy of ZD1839 and OSI-774, either alone or in combination with traditional endocrine agents (anastrozole, fulvestrant) and cytotoxic drugs in MBC patients. It has been shown that ZD1839 inhibits the growth of HER-2-overexpressing BC cell lines both *in vivo* and *in vitro* [149–151]. Moreover, a synergic growth inhibition is also observed when these cell lines are treated with the combination of trastuzumab and ZD1839 [152]. These data provide a strong rationale for a number of ongoing and planned clinical trials that intend to target the two receptors simultaneously.

EKB 569, another EGFR TKI, has been evaluated in a phase I trial [153]. PKI166, a dual inhibitor, has recently completed phase I evaluation in MBC [154–156]. Another dual inhibitor, GW 2016, has been shown to inhibit phosphorylation of HER-2 and EGFR TKs and the activation of downstream MAPK and Akt pathways in BC cell lines [157–159]. The safety, tolerability and pharmacokinetics of escalating oral doses of this agent have been assessed in a double blind, randomized, placebo controlled, crossover phase I study in healthy volunteers [160].

CI 1033, an orally active pan-erbB inhibitor, has been shown to be well tolerated and to have a favorable pharmacokinetic profile in a phase I study, in patients with advanced solid tumors [161, 162], and is currently being evaluated in a phase II MBC trial.

Many other potentially effective TKIs are awaiting clinical evaluation.

(d) *Antisense strategies.* EGFR activity can be inhibited at the nuclear level by the use of antisense oligonucleotides or ribozymes. Antisense oligonucleotides specific for EGFR and EGFR ligands interact with the complementary nucleic acid sequences in the target cell and block the transcription of specific target proteins [163]. Ribozymes are catalytically active small RNA molecules, capable of causing a site-specific cleavage of target mRNA. The interruption of gene expression by ribozymes is particularly promising for mutant forms of EGFR [164]. So far neither of these strategies has been clinically investigated.

(e) *'Combi-targeting' concept.* Another interesting approach is the 'combi-targeting' concept that involves the synthesis of chimeric compounds possessing both TK inhibiting and cytotoxic activity. Such compounds, upon hydrolytic cleavage in physiological conditions, generate a cytotoxic DNA alkylating agent and an EGFR TK inhibitor [165–167]. Three of such compounds have recently been reported to have potent antitumor activity preclinically. The rationale of this concept is based on the accumulating evidence that the antitumor activity of several chemotherapeutic agents can be enhanced when they are combined with anti-EGFR agents.

(f) *Vaccination strategies.* Patients with advanced stage tumors were immunized with human recombinant EGF chemically linked to either TT (titanic toxoid) or P64k (*Neisseria meningitidis* recombinant protein), intradermally, in a pilot clinical trial. This vaccination strategy was shown to be safe (without skin toxicity), feasible and immunogenic in humans, and to induce subjective

clinical improvement during the period when anti-EGF antibody titers were present [168].

III. Agents targeting downstream effectors of the EGFR pathway

Signal transduction pathways, which serve as 'signal-processing units', transfer signals from the cell surface to the nucleus. A number of interesting agents targeting critical elements in these STPs are in various phases of drug development.

(1) *Agents targeting RAS–RAF–MAPK pathway.* In ~30% of human cancers, mutated Ras genes produce abnormal proteins that remain locked in the activated state, thereby relaying uncontrolled proliferative signals [11]. Ras mutations are not common in BC (<5%). However, the fact that Ras can be activated by multiple stimuli, including EGFR family TKs, renders Ras a promising therapeutic target in BC [169]. Only a few agents targeting the Ras–Raf–MAPK pathway are in clinical stages of development in BC, but many of these agents are in the late phases of clinical development in other cancer types.

(a) Targeting Ras. Farnesyl transferase (FTase), which catalyzes a critical step in Ras activation, represents an important target for drug development. The initial early clinical studies with farnesyl transferase inhibitors (FTIs) focusing on colorectal cancer (CRC), NSCLC and pancreatic cancers, known to have a high incidence of K-ras mutations, did not satisfy the high expectations of therapeutic success. Surprisingly, the FTIs showed activity in some tumor types with low incidence of Ras mutations such as BC, gliomas and acute myeloid leukemia. Furthermore, emerging biological data suggest that K-ras may also be prenylated by geranylgeranyl transferase (GGTase) as an alternative to prenylation by FTase, and that the FTases can modify many other proteins involved in carcinogenesis such as RhoB, the PI3K/Akt pathway and centromere proteins E and F [11]. All these data generated suspicion with respect to the role of RAS as the principal target of FTIs. The future development of FTIs will require a revision and clarification of previously described preclinical mechanisms by which FTIs induce their biological effects in tumor tissues, and based on this improved understanding, the conduct of clinical trials with proper clinical and biological end points.

Among the multiple FTIs developed, only four reached clinical stages of evaluation. Two, R115777 (tipifarnib) and SCH 66336, are orally bio-available and the other two, BMS-214662 and L778,123, must be administered intravenously. R115777 is in various stages of development in multiple tumor types, e.g. in phase III trials in CRC and pancreatic cancer in combination with cytotoxic agents [170]. It is the only member of FTIs that is in late stages of clinical development in BC. The results of the first phase II study in ABC suggest that this agent has clinical activity with good tolerability and that the response is not dependent on ER or HER-2 status [8]. Further results on the correlation of response with EGFR expression and Ras mutation status are awaited with interest. SCH 66336 (lonafarnib) is being evaluated in a phase II trial for treatment of patients with progressive glioblastoma multiforme and in a phase III trial in advanced NSCLC patients [170]. L-778,123, which inhibits both FTase and GGTase type I, has recently been evaluated in patients with NSCLC in a phase II trial

[171, 172]. BMS 214662 is in phase I clinical trials at the moment [173, 174].

ISIS 2503 is a 20-base antisense phosphorothioate oligodeoxyribonucleotide that specifically reduces the expression of H-ras mRNA and inhibits tumor cell growth in preclinical studies [170]. The phase I data indicate a tolerable toxicity profile [175], a phase II trial has already evaluated the efficacy of this agent in advanced NSCLC patients and phase I combination studies with cytotoxic agents are currently ongoing [176, 177]. To date, this agent has not been clinically evaluated in BC.

Clinical trials evaluating the efficacy of vaccines against mutant Ras peptides are ongoing, especially in CRC and pancreatic cancer [178]. No clinical data are available yet with respect to their use in BC.

(b) Targeting Raf. BAY 439006, which inhibits Raf kinase (Raf-1), one of the downstream effectors of Ras, is the first compound of this category to enter clinical trials [179]. Available phase I data have shown that it has activity in renal, colon, pancreatic, hepatocarcinoma, ovarian and lung tumors, and could induce meaningful disease stabilizations [180]. The main toxicity of this orally bio-available agent is confined to skin including rash, palmar plantar erythema and folliculitis [170, 181]. ISIS 5132 is an antisense oligonucleotide that inhibits Raf-kinase. Phase II studies evaluating the efficacy of this agent are underway in prostate, CRC and ovarian cancers [170]. Both these agents have not yet been clinically tested in BC.

(c) Targeting MEK. CI 1040 (PD 1843220), a small molecule MEK inhibitor, has shown low toxicity and moderate efficacy in phase I trials [182, 183]. Based on this promising phase I data, this agent is moving into phase II clinical trials with 800 mg twice daily as the recommended dose.

(2) *Agents targeting PI3K/Akt pathway.* (a) Targeting mTOR. The mTOR pathway is a key growth factor-mediated signal transduction pathway that regulates cell growth, closely related to the PI3K/Akt pathway. mTOR-dependent growth factor signaling include estrogen, HER-2/neu and IGF-1, all of which can be inhibited by mTOR inhibition, resulting in G₁ arrest of the cell cycle [184]. CCI-779 is an m-TOR inhibitor with activity seen in BC cells, both *in vitro* and *in vivo*, and is the only agent of this category in later stages of clinical development in BC. PTEN-deficient, estrogen-dependent and HER-2-positive BC cell lines seem to be particularly sensitive to CCI-779. Furthermore, CCI-779 acts synergically with taxanes, doxorubicin, trastuzumab and EGFR inhibitors in BC growth inhibition. Phase I single-agent trials showed good tolerability and potential activity in breast, renal and prostate cancer. Phase II single-agent trials in breast and renal cancer were recently closed [185, 186]. The phase II BC trial evaluated two doses levels of CCI-779 (75 and 250 mg) in 109 patients with advanced disease who had failed prior chemotherapy with taxanes, anthracyclines or both. The interim report involved the first 85 patients and objective responses were seen at the two dose levels (one CR and three PRs). Side-effects were generally mild and the most frequently occurring grade 3–4 toxicities were asthenia, depression, γ glutamyl transpeptidase increase, hypercholesterolemia, leucopenia, mucositis and somnolence. Based on this preliminary clinical activity, further evaluation

Table 2. Agents targeting the downstream effectors of EGFR family (in preclinical and clinical phases of development)

Target	Agent	Mechanism of action	Phase of development	Surrogate markers evaluated	Toxicity in clinical trials
RAS–RAF–MAPK pathway	ISIS 2503	Ras antisense	Phase II – NSCLC	NA	Fever, fatigue, nausea, moderate thrombocytopenia
	R 115777 (Zarnestra®) p.o.	Farnesyl transferase inhibitors	Phase II – breast and renal cancer and MDS; phase III – colon cancer; phase II/III – pancreas; phase I – leukemias, NSCLC; phase I/II HG astrocytoma	Buccal mucosa prelamin A	Myelosuppression, fatigue, rash, neuropathy, diarrhea, nausea, hypotension, vomiting, headache (DLT in phase I: neuropathy, fatigue, neutropenia)
	BMS 214662		Phase I	Rho-B, lamin B, ras in PBL; FT activity in PBMCs	Nausea, vomiting, diarrhea, fatigue, elevation of transaminases
	SCH 66336 p.o.		Phase III – NSCLC; phase II – GM	Peroxisomal protein (PxF) in PBMC and prelamin A in buccal mucosa	Nausea, vomiting, fatigue, diarrhea, headache, moderate myelosuppression
	L-778,123		Phase II – NSCLC	HDJ-2, Rap1A; in PBMC	Nausea, vomiting fatigue, myelosuppression, QT prolongation
	BAY 439006 p.o.	Raf kinase inhibitor	Phase I	ERK1/2 in PBL by FC	Palmar–plantar erythema, anorexia, fatigue, alopecia, and diarrhoea
	ISIS 5132	Raf antisense	Phase II – prostate and colon	NA	Fever
	CI 1040 (PD 184352)	MEK inhibitor	Phase I completed	pMAPK in PBMC and tumor	Diarrhea, fatigue, nausea, rash, vomiting
PI3K–Akt pathway	CCI 779	mTOR inhibitor	Phase II – BC; phase II – renal cancer	PTEN, Akt	Hyperglycemia, anaemia hypertriglyceridemia, hypokalemia, leucopenia, mucositis, elevated GGT
	Wortmanin	PI3K inhibitors	Preclinical – BC cell lines	NA	NA
	LY 294002		Preclinical – BC cell lines	NA	NA
	17-AAG	HSP90 inhibitor	Phase I completed	Lck kinase, Akt, Raf-1, HSP 70, HSP Grp 78, CDK4 in PBLs, HER-2 and MAPK in skin, changes in 18-FDG by PET scan	Fatigue, nausea, vomiting, hepatotoxicity

EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; p.o., orally; NSCLC, non-small-cell lung cancer; HG, high grade; GM, glioblastoma multiforme; FC, flow cytometry; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; Lck (p56^{lck}), protein from the Src family of tyrosine kinases; Grp 78, 78 kDa glucose-regulated protein that belongs to the family of ~70 kDa heat shock proteins (HSP70); HDJ-2, HSP40; DLT, dose-limiting toxicity; NA, not available; GGT, γ glutamyl transpeptidase; FDG, fluorodeoxyglucose; PET, positron emission tomography; Rap 1A, Ras-related protein 1A; BC, breast cancer.

of this agent in combination with other treatment modalities is planned in BC (187).

(b) Targeting Akt. 17-AAG, an indirect inhibitor of Akt activity, is an ansamycin antibiotic which has shown antitumor effects in HER-2-overexpressing BC cell lines in several preclinical experiments [60, 61]. It inhibits the chaperone function of HSP90, which in turn depletes several key signaling proteins involved in cell cycle control, hormone signaling and growth factor pathways, including HER-2, Akt and Raf-1. The preliminary findings from phase I trials in advanced cancer patients reported disease stabilization with acceptable toxicity [118, 187].

PTEN gene therapy is among other attractive strategies aimed at inhibiting Akt activity, since mutations of this gene by themselves lead to cancer syndromes, and because Akt has increasingly been shown to play an important role in BC [76, 188].

Surrogate markers for measuring the biological effects of targeted agents

As stated in the introductory part of this review, one of the most important issues in trials evaluating the efficacy of targeted biological agents is the development of assays to measure the desired

biological effect. The development of such assays requires a clear understanding of the mechanism of action of the drug under investigation in order to select the most appropriate marker of therapeutic activity. The practical limitations of tumor biopsies, such as availability and accessibility, are critical issues. Therefore, the development of assays in surrogate tissues such as skin and blood cells, which normally express the target molecules and are easily accessible, offers an important opportunity to observe the biological activity of these agents. However, it is unknown to what extent the changes in the surrogate tissues reflect the effects of the agent in the tumor. In other words, the demonstration of inhibition of a surrogate end point does not necessarily indicate a treatment effect in the tumor. Studies comparing biopsies from both surrogate tissues and tumor tissues are needed. In addition, it is essential to correlate clinical outcome and surrogate markers prospectively, in order to validate their predictive value. Tables 1 and 2 summarize the surrogate markers and the sites measured in clinical trials testing anti-EGFR agents. Consequently, on the eve of cancer therapy driven by proteogenomic information, obtaining frozen samples of all tumors for future evaluation is of utmost importance.

Conclusions

Given this wide range of new attractive molecules, the future holds promise for witnessing major advances in the management of BC. So far, continuing enthusiasm for the identification of new, targeted agents has removed the blindfold from our eyes. We are no longer playing blind man's bluff, no longer running in the dark. However, the clarification of whether the current state of excitement in the development of new targeted biological agents will be accompanied by a therapeutic benefit, as in the case of trastuzumab and STI 571, is the second essential step of our job. This could only be achieved by making tremendous efforts to evaluate these new molecules in well designed randomized clinical trials.

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