OPINION

Targeting the ERBB family in cancer: couples therapy

Niall Tebbutt, Mikkel W. Pedersen and Terrance G. Johns

Abstract | The ERBB family of receptor tyrosine kinases has a central role in the tumorigenesis of many types of solid tumour. Various therapeutics targeting these receptors have been approved for the treatment of several cancers. Considerable preclinical data have shown that the administration of two inhibitors against an individual ERBB family member — particularly epidermal growth factor receptor (EGFR) or ERBB2 — leads to markedly higher antitumour activity than the administration of single agents. This Opinion article describes the preclinical and clinical performance of these dual-targeting approaches, discusses the key mechanisms that mediate their increased efficacy and highlights areas for ongoing investigation.

Members of the ERBB family of receptors are expressed in many cells of the epithelial, mesenchymal and neuronal lineages, in which they have diverse roles in development, proliferation and differentiation[1]. All four members of the ERBB family are receptor tyrosine kinases (RTKs) with an analogous structure, consisting of an extracellular ligand-binding domain, a single hydrophobic transmembrane region and an intracellular segment that contains a conserved tyrosine kinase domain[2]. These receptors interact with a family of 12 polypeptide growth factors, the binding of which stimulates both homodimeric and heterodimeric interactions between family members, leading to autophosphorylation of a number of cytoplasmic tyrosine residues[3]. These phosphorylated tyrosine residues serve as docking sites for many adaptor and signalling proteins that mediate the complex and diverse responses generated by receptor activation[4]. ERBB family receptors activate several downstream pathways, including the RAS–ERK and PI3K–AKT pathways. Epidermal growth factor receptor (EGFR; also known as ERBB1) and ERBB4 are classical RTKs. By contrast, ERBB2 (also known as HER2) is unable to bind any known ligand, and ERBB3 is generally considered to be kinase-deficient[1–4], although a recent study showed that ERBB3 can bind ATP and has a low level of kinase activity[5]. Therefore, the activation of both ERBB2 and ERBB3 requires their heterodimerization with other family members. Heterodimerization creates additional signalling diversity and amplification of the response among ERBB family members. Interestingly, the most potent mitogenic signals are created by ERBB2–ERBB3 heterodimers[6].

Aberrant tyrosine kinase activity of ERBB family members can result in unregulated growth stimulation and tumorigenesis in various tumour types, including breast, lung, brain, head and neck, and colon tumours[7]. Inappropriate activation of EGFR and ERBB2 in cancer can occur through a range of mechanisms, including overexpression (often due to gene amplification), point mutations, partial deletions and autocrine ligand–receptor stimulation[8]. Overexpression and/or mutation can lead to ligand-independent activation of EGFR and ERBB2, as well as to increased activation following engagement with the ligand. Owing to its lack of kinase activity, the oncogenic function of ERBB3 is predominantly mediated through overexpression and interaction with EGFR or ERBB2 (REF. 8). The role of ERBB4 is more complex because it has multiple isoforms with differing activities, some that seem to be oncogenic and others that seem to be tumour suppressive[9]. However, the recent observation that point mutations in *ERBB4* are present at low levels in several tumour types has definitively confirmed that activated ERBB4 can be pro-tumorigenic[10].

The frequent activation of ERBB family members in cancer makes them attractive therapeutic targets. As EGFR and ERBB2 are the family members with the best-defined roles in cancer, most drug development programmes and clinical trials have been based on targeting these receptors; therefore, we focus our discussion on targeting these proteins. Although most strategies have used these drugs individually, in this Opinion article, we describe how over the past 10 years, extensive preclinical studies and initial clinical data suggest that targeting either EGFR or ERBB2 combinatorially — by using two different antibodies, or an antibody and a tyrosine kinase inhibitor (TKI) — has additive or even synergistic antitumour activity. We discuss the mechanisms that lead to this synergy and describe how this strategy is being translated to the clinic.

EGFR- and ERBB2-targeted therapies

Various small-molecule TKIs directed to EGFR have been developed over the past 30 years[7] (FIG. 1), and many preclinical *in vitro* and animal studies have indicated that these molecules should have considerable promise in the treatment of solid tumours[11,12]. First-generation EGFR TKIs, such as erlotinib and gefitinib, are reversible inhibitors that compete for kinase domain binding with endogenous ATP[7], thus preventing its tyrosine-phosphorylating activity and blocking downstream signalling. These agents are the 'poster child' for targeted therapy for solid tumours: a subset of patients with non-small-cell lung cancer (NSCLC) that contains mutations in the kinase domain of EGFR respond well to EGFR TKIs. However, this finding is the exception because EGFR-targeted TKIs have generally performed poorly as single agents[13,14]. Afatinib is a second-generation EGFR TKI that binds irreversibly to the free cysteine in the kinase domain of EGFR, but its efficacy has not yet been comprehensively evaluated.

EGFR-targeted antibodies that prevent ligand binding (such as cetuximab and panitumumab) have also been developed and are approved in several countries for the treatment of colon cancer, as well as head and neck cancer[12] (FIG. 1; TABLE 1). EGFR TKIs directly bind to the kinase domain and block its kinase activity, whereas EGFR-targeted antibodies bind extracellularly, blocking ligand binding and, in some cases, preventing receptor dimerization[12]. The responses to EGFR-targeted antibodies are relatively low, with improvements in survival usually lasting only several months, and efficacy is again restricted to certain patient subtypes[15]. In particular, only patients with colon cancer whose tumours are *KRAS* wild-type seem to respond to EGFR-targeted antibodies[15]. Recently, mixtures of new EGFR-targeted antibodies — including Sym004 and MM-151— have been developed, and these target non-overlapping epitopes on EGFR.

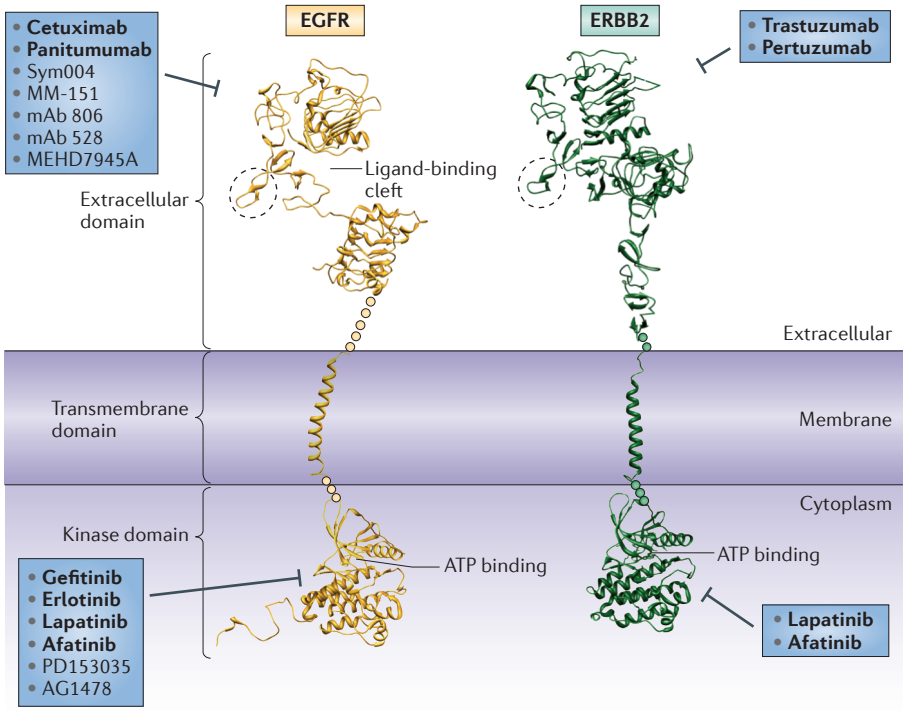

Figure 1 | **EGFR and ERBB2 structure and therapeutic targets.** Both epidermal growth factor receptor (EGFR) and ERBB2 have an extracellular domain, a single transmembrane domain and an intracellular kinase domain. EGFR has a ligand-binding cleft in its extracellular domain, and this cleft interacts with a number of ligands, including EGF and transforming growth factor-α (TGFα). In the presence of ligand, dimers form through interactions between the dimerization loop (dashed oval) on each monomer. Dimerization results in the activation of the kinase domain and the initiation of intracellular signalling pathways (FIG. 2). ERBB2 does not bind any known ligand and usually needs to form heterodimers with other members of the ERBB family to be activated, although overexpression of ERBB2 can lead to the formation and activation of homodimers. Given their role in tumorigenesis, EGFR and ERBB2 are attractive therapeutic targets in various cancers. Therapeutic antibodies are directed to the extracellular domains of EGFR and ERBB2 and tyrosine kinase inhibitors (TKIs) to the kinase domains (as listed in blue boxes). Drugs that have been approved for the clinic are indicated in bold. Afatinib has been granted priority review for approval by the US Food and Drug Administration in 2013. Yellow and green circles indicate segments for which the crystal structures have not yet been solved. Figure is modified, with permission, from REF. 7 © (2012) Macmillan Publishers Ltd. All rights reserved.

For ERBB2, no specific TKIs have been described, although lapatinib is a TKI that inhibits both EGFR and ERBB2. ERBB2-targeted antibodies include trastuzumab and pertuzumab. As ERBB2 has no ligand, antibodies specific for this receptor can inhibit activity through mechanisms such as preventing dimerization[16], although the exact mechanism by which some ERBB2-targeted antibodies function is unknown.

Preclinical dual ERBB targeting

In the earliest report describing the use of two inhibitors directed against the same receptor (dual targeting), it was shown that a small-molecule EGFR-specific TKI (PD153035) and the EGFR-specific antibody C225 (now known as cetuximab) had additive antiproliferative activity *in vitro* against A431 epidermoid carcinoma cells, which contain an amplified *EGFR* gene[17]. This dual inhibitor concept was then validated in xenograft models against several cancer cell lines, using the EGFR-targeted monoclonal antibody 806 (mAb 806) and the prototypical EGFR-specific TKI AG1478; indeed, synergistic inhibition of xenograft growth was observed in a model of head and neck cancer using HN5 cells, which also have an amplified *EGFR* gene[18]. The synergy between these two EGFR-targeting agents was later extended to A431 xenografts[19]. Since then, this dual targeting approach has been confirmed and extended[20–23].

The combination of cetuximab and gefitinib was shown to have synergistic *in vitro* antiproliferative activity against A431 cells and additive activity against a range of other cancer cell lines[21]. This combination was also synergistic when used to treat A431 xenografts, with the dual treatment inducing complete regressions, which were not seen in the single-treatment groups. Cetuximab used in combination with either gefitinib or erlotinib showed greater than additive antiproliferative activity against a range of head and neck cancer cell lines and lung cancer cell lines *in vitro* that expressed different levels of EGFR[22]. Both combinations also had more than additive antitumour activity in an H226 lung cancer xenograft model, which overexpresses EGFR. Dual treatment of the biliary tract cancer cell line HuCCT1, which overexpresses EGFR, with cetuximab and erlotinib induced greater than additive apoptosis both *in vitro* and in xenograft models[23].

More recently, the ERBB2-targeted antibody trastuzumab in combination with lapatinib has been shown to inhibit the *in vitro* and xenograft growth of human breast cancer cell lines significantly more than either agent alone[24]. In particular, dual treatment with trastuzumab and lapatinib caused complete regression of BT474 breast cancer xenografts, which have an amplified *ERBB2* gene, whereas single-agent treatment caused only partial tumour regression. These studies were extended to an MCF7 breast cancer xenograft model expressing transfected *ERBB2* (REF. 25). This combination of ERBB2 inhibitors had synergistic *in vitro* antiproliferative activity against gastric cancer cell lines and enhanced apoptosis compared with single-agent treatment[26]. N87 gastric xenografts (which contain amplified *ERBB2*) that were treated with trastuzumab in combination with lapatinib also showed more complete regression than those treated with single agents[26].

The use of two antibodies directed towards EGFR or ERBB2 also has higher antitumour activity in a range of cancer models than single agents. The combination of two EGFR-targeted antibodies, mAb 806 and mAb 528, displayed synergistic antitumour activity against A431 xenografts and U87MG glioma xenografts transfected with a constitutively active EGFR mutant[20]. Dual targeting of ERBB2 by trastuzumab and pertuzumab showed synergistic inhibition of tumour growth in both lung and breast cancer xenograft models[27]. An additional study showed that this antibody combination had synergistic antitumour activity against two gastric cancer xenograft models[28].

Mechanisms of increased efficacy

The targeting of EGFR signalling with two inhibitors leads to more efficient inhibition of the receptor by several mechanisms. As discussed in detail below, these include increased receptor inhibition, blockade of

Table 1 | **Single-agent activity of EGFR and ERBB2 inhibitors**

Inhibitor	Reagent type*	Development stage	ERBB-binding site	Notes on the mechanism of action
EGFR				
Cetuximab	Antibody	Approved	Ligand-binding site in domain III	Blocks ligand binding and impedes dimerization[16]. Mediates ADCC[79]
Panitumumab	Antibody	Approved	Ligand-binding site in domain III	Blocks ligand binding and impedes dimerization[16]. Mediates ADCC[80]
mAb 806	Antibody	Clinical trial	Residues 287–302 in domain II	Epitope is only transiently exposed as EGFR moves from its inactive to its active state[81,82], an event that is generally restricted to cancer cells. Inhibits EGFR activity, but the exact mechanism is unknown
mAb 528	Antibody	Experimental	Ligand-binding site in domain III	Blocks ligand binding and impedes dimerization[16]
Gefitinib	TKI	Approved	Kinase domain (preferentially binds kinase-active conformation)	Inhibits kinase activity by competing reversibly with ATP. Most active against kinase domain mutants expressed in NSCLC[7,83,84]
Erlotinib	TKI	Approved	Kinase domain (preferentially binds kinase-active conformation)	Inhibits kinase activity by competing reversibly with ATP. Most active against kinase domain mutants expressed in NSCLC[7,83,84]
PD153035	TKI	Experimental	Kinase domain	Inhibits kinase activity by competing reversibly with ATP[85]
AG1478	TKI	Experimental	Kinase domain	Inhibits kinase activity by competing reversibly with ATP[18]
ERBB2				
Trastuzumab	Antibody	Approved	Domain IV	Suggested modes of action include impeding dimerization, increasing endocytic destruction, inhibiting receptor cleavage and mediating ADCC activity; the relative contribution of these mechanisms in patients is uncertain[16,86]
Pertuzumab	Antibody	Approved	Dimerization loop	Inhibits homodimerization and heterodimerization[16]. Mediates ADCC[28]
EGFR and ERBB2				
Lapatinib	TKI	Approved	Kinase domain (preferentially binds kinase-inactive conformation of EGFR)	Inhibits kinase activity of both receptors by competing reversibly with ATP[87,88]
Afatinib	TKI	Clinical trial	Kinase domain	Inhibits kinase activity of both receptors by competing irreversibly with ATP[89,90]

ADCC, antibody-dependent cell cytotoxicity; EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; NSCLC, non-small-cell lung cancer; TKI, tyrosine kinase inhibitor. *As a class, ERBB TKIs increase the cell-surface expression of their target receptors by decreasing receptor internalization[19,24].

additional signalling pathways, prevention of ERBB heterodimerization, changes in receptor conformation, enhanced antibody-dependent cell cytotoxicity (ADCC) and receptor downregulation (FIGS 2–4). The most generally applicable of these mechanisms is the enhanced inhibition of EGFR kinase activity, which leads to reduced downstream signalling[20,24,29].

Antibody–TKI combinations that enhance inhibition. The combined use of an antibody and a TKI to target EGFR results in more effective inhibition of kinase activity than the use of single agents. Indeed, more efficient blockade of EGFR signalling using the combination of various EGFR-targeted antibodies and reversible TKIs (see below) (FIG. 2a) has been observed in a range of tumour xenograft models and in a lung cancer transgenic model[19–23,29,30]. Likewise, compared with single agents, more efficient inhibition of ERBB2 with the combination of trastuzumab and lapatinib has been reported in gastric and breast cancer xenograft models[24–26].

The different mechanisms of action of TKIs and antibodies may contribute to this enhanced inhibition. For example, because erlotinib and gefitinib are reversible inhibitors[7], if the TKI is replaced by ATP at the cell surface, there is the potential for EGFR reactivation. The co-binding of antibody reduces this reactivation by preventing ligand-induced or ligand-independent dimerization, leading to more sustained EGFR inhibition, and this is one of the important ways in which dual targeting causes greater EGFR inhibition[19]. Different threshold levels of EGFR activation lead to alterations in the range of downstream targets that are phosphorylated by EGFR; a higher level of activation activates more pathways[31]. Thus, the more efficient inhibition of EGFR that is generated by the use of dual targeting not only leads to the more efficient blockade of key downstream signalling pathways (such as the AKT and ERK pathways) but also leads to the blockade of a greater range of signalling pathways (FIG. 2a). Indeed, the combination of cetuximab and erlotinib inhibits a greater range of downstream pathways than the single agents in colon cancer cells *in vitro*[29].

Prevention of heterodimerization. There is considerable heterodimerization and crosstalk between all four members of the ERBB family[2–4]. As ERBB2 cannot bind ligand, it needs to heterodimerize with other ERBB family members to be activated, unless it is substantially overexpressed[32]. ERBB3 has a kinase domain with limited or no activity and therefore needs to heterodimerize with other ERBB proteins to become phosphorylated and signal[6]. The activation of non-targeted ERBB family members can reduce the effectiveness of both EGFR- and ERBB2-targeted therapies[33–35]. Inhibition of EGFR and ERBB2 by lapatinib, in combination with an antibody such as trastuzumab, is one strategy for reducing crosstalk between ERBB family members (FIG. 2b). This approach has been shown to reduce the heterodimerization of ERBB2 with EGFR and with ERBB3 in breast cancer cell

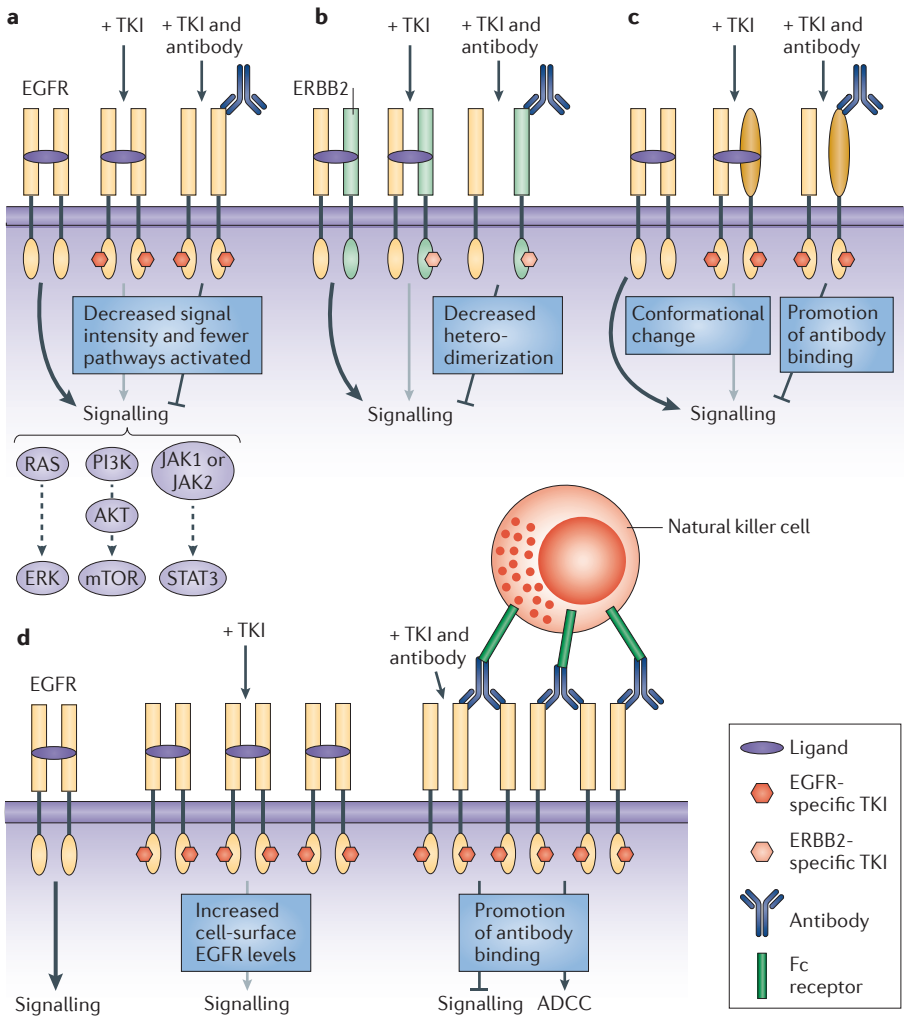

Figure 2 | **Enhanced antitumour mechanisms mediated by dual EGFR therapy.** Dual therapy with a tyrosine kinase inhibitor (TKI) and a specific antibody enhances antitumour activity by several mechanisms. Some major mechanisms have been demonstrated, including decreased signal intensity (part **a**); decreased receptor heterodimerization (part **b**); induction of a conformational change in the receptor (part **c**); and induction of antibody-dependent cell cytotoxicity (ADCC) (part **d**). Ligand-bound epidermal growth factor receptor (EGFR) (part **a**) homodimerizes and becomes autoactivated, leading to downstream signalling through a variety of pathways that can drive cancer growth and survival. Reversible EGFR-specific TKIs bind to the ATP pocket of EGFR, substantially reducing signalling. However, these TKIs can be replaced by ATP at the binding site, resulting in sustained signalling at low levels. The addition of an EGFR-targeted antibody, in combination with the TKI, inhibits such EGFR activation by preventing ligand binding and EGFR dimerization. Thus, even if the TKI is replaced by ATP, the receptor remains unable to signal. Consequently, this dual inhibition is more effective at preventing EGFR signalling and blocks a greater range of pathways than either agent used alone (several representative signalling pathways are indicated). This is probably the main mechanism associated with the use of dual therapeutics. EGFR and ERBB2 (part **b**) can heterodimerize, diversifying and increasing signalling compared with that from EGFR homodimers. The addition of a TKI specific for either receptor reduces signalling from the heterodimer but does not directly prevent dimerization. Adding an EGFR-targeted or ERBB2-targeted antibody in combination with the TKI directly reduces heterodimerization and prevents downstream signalling. EGFR-specific TKIs (part **c**) can change the conformation of EGFR (shown as dark yellow ovals), increasing the binding of certain EGFR-targeted antibodies and thereby making them more efficient at inhibiting the receptor. TKIs directed towards EGFR (part **d**) cause retention of the receptor on the cell surface, resulting in increased levels of cell-surface EGFR-targeted antibody. This higher concentration of surface antibody interacts and activates natural killer cells more efficiently, stimulating a stronger ADCC response to the cancer cell. Thick black arrows indicate strong signalling, thin grey arrows indicate weak signalling, inhibiting arrows indicate no signalling and dashed arrows indicate multiple steps. Although the details are different, dual antibody therapy also functions through these mechanisms; however, the use of two antibodies decreases rather than increases cell-surface expression of the ERBB receptors. JAK, Janus kinase; STAT, signal transducer and activator of transcription.

lines harbouring amplified *ERBB2* (REF. 24). Interestingly, a recent study showed that this same combination of inhibitors stimulates a subsequent increase in ERBB3 expression, which helps to restore ERBB2–ERBB3 heterodimerization, preventing full signalling inhibition even in the presence of two inhibitors[35].

The enhanced antitumour activity when using two ERBB2-targeted antibodies, trastuzumab and pertuzumab, was shown to be partly due to a marked reduction in heterodimerization of ERBB2 with both EGFR and ERBB3, leading to less phosphorylation and less activation of both receptors[28]. This work suggests that dual antibody approaches directed towards ERBB2 may be more efficient at preventing ERBB cross-talk than antibody and TKI combinations, although additional studies are required to confirm this.

EGFR-specific antibody–TKI combinations that alter receptor conformation and glycosylation. The early demonstration of a synergistic antitumour response using the EGFR-targeted antibody mAb 806 and the EGFR-specific TKI AG1478 also showed that AG1478 induced a change in the conformation of EGFR, thus stimulating the formation of an inactive dimer to which mAb 806 bound with greater affinity[18] (FIG. 2c). Furthermore, long-term treatment with AG1478 altered the glycosylation of the cell-surface EGFR in a manner that also increased mAb 806 binding[19].

Antibody–TKI combinations that increase surface receptor expression and ADCC. In addition to altering EGFR conformation and glycosylation, AG1478 increased the cell-surface expression of EGFR *in vitro* by preventing receptor internalization in A431 epidermoid carcinoma cells that overexpress the receptor because of an amplified *EGFR* gene[19]. This increase in cell-surface EGFR, and hence increase in the cell-surface EGFR-targeted antibody mAb 806, may increase ADCC activity, an observation that was later confirmed using cetuximab in combination with erlotinib against lung cancer cells expressing EGFR[36]. Thus, TKIs can increase antibody binding to cell-surface EGFR by a range of mechanisms, leading to more efficient receptor inhibition and signalling blockade and possibly increasing ADCC activity against target cells (FIG. 2c,d).

The EGFR and ERBB2 TKI lapatinib increases the amount of ERBB2 on the surface of breast cancer cells that overexpress

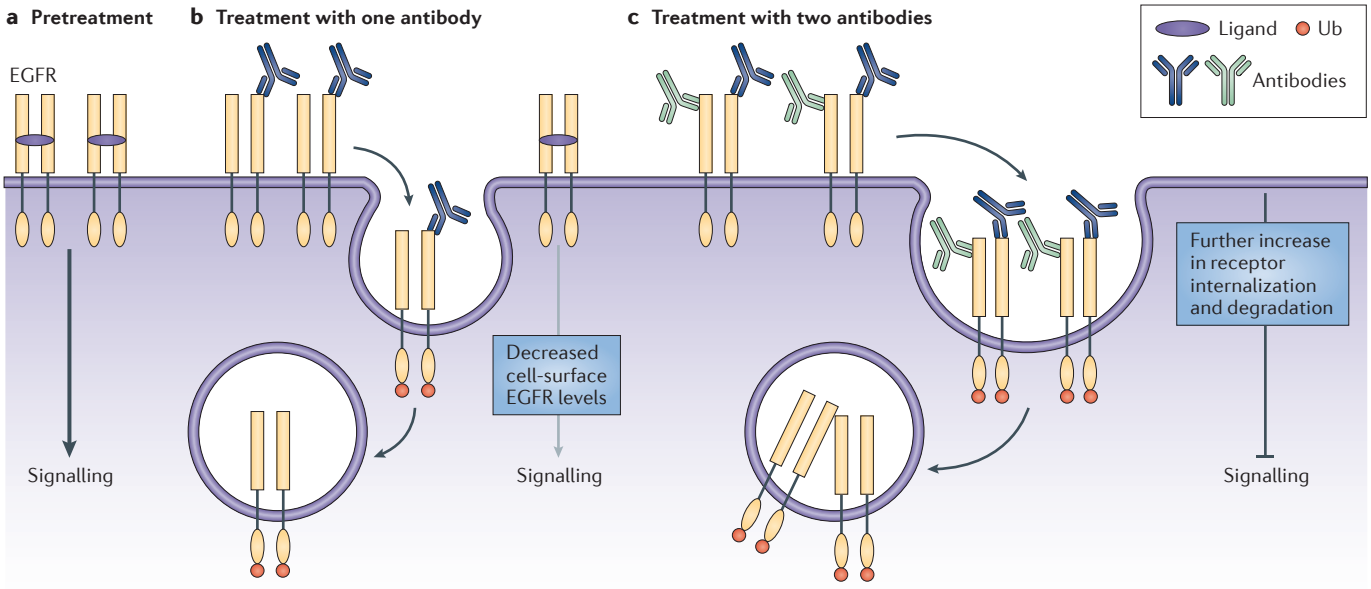

Figure 3 | **Dual antibody therapy increases EGFR degradation.** The binding of ligand to epidermal growth factor receptor (EGFR) (part **a**) is blocked by most EGFR-targeted antibodies (part **b**). These antibody–EGFR complexes are internalized slowly into the cell, where EGFR can be ubiquitylated (Ub) and targeted for lysosomal degradation. However, some EGFR molecules remain on the cell surface, mediating persistent signalling (part **b**). Addition of a second EGFR-targeted antibody that binds to a non-overlapping epitope promotes the crosslinking of EGFR, stimulating increased internalization and degradation of the receptor (part **c**). Consequently, the amount of cell-surface EGFR is markedly reduced, causing a further reduction in receptor signalling. Thick black arrows indicate strong signalling; thin grey arrows indicate weak signalling; and inhibiting arrows indicate no signalling.

ERBB2 by preventing receptor internalization both *in vitro* and in a xenograft model using BT474 cells. This, in turn, increases the number of non-active ERBB2 homodimers and ERBB2–ERBB3 heterodimers[24]. By contrast, trastuzumab activates ERBB2, leading to its internalization and degradation[24]. Importantly, trastuzumab cannot induce ERBB2 internalization when used in combination with lapatinib, leading to an increase in the amount of antibody on the cell surface, in both the *in vitro* and xenograft models. This higher surface density of trastuzumab leads to a significant increase in ADCC[24] (FIG. 2d). Given that the EGFR-targeted antibody cetuximab and the ERBB2-targeted antibody trastuzumab both mediate some of their antitumour activity through ADCC[37,38], these observations on the combination of antibody and TKI are clinically important. Recently, two groups have shown independently that in genetic mouse models of breast cancer driven by *Erbb2*, murine antibodies directed towards ERBB2 mediate part of their antitumour activity through T-cell-dependent immune responses that involve both $CD4^+$ and $CD8^+$ T cells[39,40]. It would be interesting to determine whether trastuzumab also stimulates T cell responses and whether the lapatinib-mediated increase in ERBB2 cell-surface expression increases this activity.

Dual antibody combinations that decrease surface receptor expression and signalling. The most important feature of dual antibody therapy is the ability to stimulate EGFR internalization and degradation, leading to a dramatic loss in cell-surface receptor[20,41] (FIG. 3). Although this is the opposite effect to the result of a combination of TKI and antibody (which increases cell-surface receptor levels), it has the same overall effect of blocking receptor activation and downstream signalling more efficiently than the use of single antibodies. This downregulation of cell-surface EGFR was first shown in glioma xenograft models expressing EGFR variant III (EGFRvIII)[20], which is a ligand-independent EGFR mutant expressed in glioma that innately has low levels of internalization[42]. Treatment with the EGFR-targeted antibodies mAb 528 or mAb 806 alone inhibited glioma xenografts expressing EGFRvIII, but neither mAb 528 nor mAb 806 alone caused EGFR downregulation. By contrast, treatment with both antibodies synergistically reduced tumour growth and stimulated a marked decrease in cell-surface EGFRvIII, even in an orthotopic xenograft model in which tumours were grown in the brain[20].

More recently, it was shown that the administration of two new antibodies with non-overlapping epitopes specific for domain III of EGFR (the domain that contains the epitope for cetuximab) removes up to 80% of the EGFR molecules from the surface of many EGFR-expressing cancer cells[41]. This downregulation of EGFR caused a marked reduction in downstream signalling and inhibited cell proliferation and migration. This combination of antibodies stimulated EGFR internalization and degradation, mostly by preventing endosomal recycling of EGFR back to the cell surface[41]. Unlike ligand-induced downregulation, EGFR was not activated by this antibody combination, leading to synergistic inhibition of the proliferation and the migration of cells secreting autocrine ligand[41]. Sym004, which is a combination of two new EGFR-targeted antibodies that bind non-overlapping epitopes on domain III, also efficiently causes EGFR downregulation by promoting receptor internalization and degradation[43].

Clearly, removing substantial amounts of EGFR from the cell surface severely curtails EGFR signalling. However, the published preclinical data suggest that removing EGFR from the surface has even more important antitumour effects than simply the inhibition of signalling. One plausible explanation is that this approach also blocks the non-kinase functions of EGFR. Functions ascribed

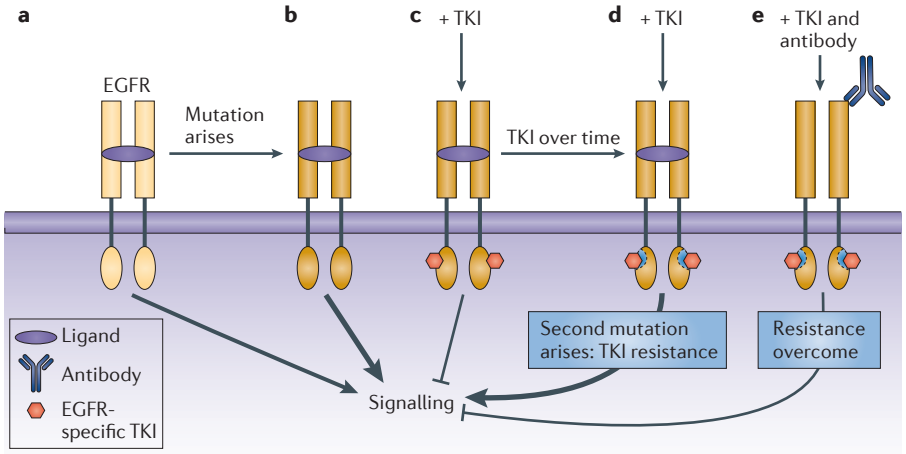

Figure 4 | **Dual EGFR therapy overcomes TKI resistance.** The usual response to ligand binding of epidermal growth factor receptor (EGFR) (part **a**) is increased in patients with lung cancer who have point mutations in the kinase domain (for example, L858R). Such mutations produce receptors that are autoactive (shown in dark yellow) (part **b**). Lung cancers expressing these mutated EGFRs are addicted to the EGFR pathway and respond to treatment with EGFR-specific tyrosine kinase inhibitors (TKIs) (part **c**). Invariably, however, patients develop resistance to EGFR TKI therapy, and the lung cancer begins to regrow. A major mechanism of resistance is the selection of cells expressing EGFR with a secondary point mutation of the kinase domain (for example, T790M; shown in blue), which decreases the binding of the TKI, leading to reactivation of EGFR signalling (part **d**). Although EGFR-targeted antibodies also have only modest antitumour activity against EGFR with double mutations, the combination of a TKI and an antibody markedly inhibits tumour growth (part **e**). The mechanism associated with this increased antitumour activity has not yet been elucidated. Thick black arrows indicate stronger signalling than thin black arrows.

to the non-activated EGFR include protection against p53-upregulated modulator of apoptosis (PUMA)-mediated apoptosis[44] and prevention of autophagy through maintenance of glucose uptake[45]. Furthermore, EGFR downregulation, but not kinase inhibition, has been shown to sensitize prostate cancer cells to chemotherapy[46]. Therefore, the downregulation of EGFR that is mediated by dual antibody therapy probably invokes additional antitumour activities that are not associated with kinase inhibition alone.

Intuitively, it would be expected that the receptor downregulation that is induced by dual antibody therapy would lead to fewer antibody molecules on the cell surface overall and, therefore, to reduced ADCC. However, although the combination of trastuzumab and pertuzumab reduced the amount of cell-surface ERBB2 on gastric cancer cells, this combination still resulted in increased *in vitro* ADCC activity[28]. This finding suggests that the binding of two antibodies per target receptor may be more important for inducing ADCC activity than the total amount of antibody on the cell surface, as it leads to more efficient activation of natural killer cells. Additional studies with two EGFR-targeted antibodies are needed to determine whether this observation is more generally applicable.

Overcoming drug-resistant receptor mutations. EGFR should be an attractive target in glioma — a particularly lethal form of brain cancer — as it is frequently overexpressed or mutated (particularly in the extracellular domain)[47,48]. However, strong clinical responses to gefitinib and erlotinib are rare in high-grade glioma[47]. In the case of gefitinib at least, this is not due to its inability to cross the blood–brain barrier[49]. The EGFRvIII extracellular-domain mutant of EGFR is expressed in 30% of high-grade gliomas[50]. This form of EGFR is resistant to some EGFR-targeted therapeutics, such as gefitinib, when they are used as single agents[51]. As discussed above, the combination of two EGFR-targeted antibodies has been shown to synergistically inhibit the growth of glioma xenografts[20] through the removal of EGFRvIII from the cell surface by an unknown mechanism. This study indicated that dual targeting may have a role in overcoming the innate resistance that is mediated by the mutation of EGFR.

Extracellular domain EGFR mutations in glioma are autoactivating and oncogenic, whereas most EGFR molecules are in the kinase-inactive conformation; however, erlotinib and gefitinib preferentially bind to the active conformation[48]. Lapatinib, which has been shown to preferentially bind to the inactive conformation of EGFR, was found to be more effective than erlotinib at inhibiting the growth of glioma cells through an EGFR-dependent mechanism[48]. Unfortunately, lapatinib does not cross the blood–brain barrier at sufficient levels to inhibit glioma growth[48]. Although the strategy has not been tested, these observations suggest that using dual TKIs, one specific for the active conformation of EGFR and the other for the inactive conformation, may also enhance antitumour activity, particularly if gefitinib can lower the dose of lapatinib that is required for the effective inhibition of growth.

Lung cancers also frequently express mutant EGFR; however, in contrast to gliomas, these cancers have various EGFR kinase domain mutations that render them sensitive to EGFR TKIs. Nevertheless, resistance invariably develops following treatment with an EGFR TKI, often as a result of secondary mutations within the kinase domain that alter the binding of the TKI[52]. The most common of these secondary mutations is T790M, which causes resistance to both gefitinib and erlotinib[52]. Using both genetic and xenograft lung cancer models, it has been shown that T790M-containing tumours were relatively resistant to both cetuximab and afatinib as single agents[30]. However, the combination of both agents caused significant tumour shrinkage, indicating that dual targeting is a possible strategy for overcoming acquired resistance[30] (FIG. 4).

Localization of receptors to organelles. Dual combination therapy may enhance antitumour activity by additional mechanisms that have not yet been studied. Following activation, EGFR can traffic to the nucleus, where it can function as a transcriptional co-activator for several oncogenes and can promote the replication and repair of DNA[53]. Importantly, increased levels of nuclear EGFR have been associated with resistance to both cetuximab[54] and gefitinib[55]. Similarly, EGFR and EGFRvIII have been shown to translocate to the mitochondria in the presence of activated SRC[56,57]. Mitochondrial EGFR and EGFRvIII can contribute to chemoresistance and have a role in regulating glucose metabolism in cancer cells. Combination EGFR therapy may prevent nuclear and/or mitochondrial localization of EGFR, thus enhancing the antitumour activity of dual targeting.

Clinical trials

Combination of an antibody and a TKI. Initial studies of dual EGFR targeting involved the combination of cetuximab with gefitinib (TABLE 2). A Phase I study in patients with NSCLC refractory to

platinum-based chemotherapy established the safety of the combination of cetuximab (250 mg m^{-2} per week) and gefitinib (250 mg per day). Although no tumour responses were observed, four of the patients had stable disease[58]. Another study reported safety and efficacy data for this combination from a Phase I study in patients with colorectal cancer (CRC), head and neck cancer or NSCLC[59]. The combination was generally tolerable at full-dose cetuximab (250 mg m^{-2} per week) and gefitinib at 500 mg per day. In addition, preliminary efficacy data demonstrated promising efficacy in patients with CRC, with an overall response rate of 56%.

Similarly designed studies have examined the combination of cetuximab and erlotinib as an alternative TKI. A Phase I study, involving a range of tumours but predominantly NSCLC, showed that cetuximab (250 mg m^{-2} per week) combined with erlotinib (150 mg per day) was tolerable[60]. A modest response rate of 8% was observed in patients with NSCLC. A Phase Ib–Phase II study evaluated the activity and safety of the combination of cetuximab (500 mg m^{-2} every second week) and erlotinib (100 mg per day) in patients with *EGFR*-mutant lung adenocarcinoma with acquired resistance to erlotinib therapy[61]. The combination treatment was tolerable at these doses; however, no marked clinical activity was observed. In contrast to these data, a similarly designed study in patients with acquired resistance to EGFR TKIs tested the combination of cetuximab with the irreversible EGFR TKI afatinib (40 mg per day)[62]. An encouraging response rate of 36% was observed, with an acceptable safety profile. Additional data using this combination are being generated.

The Phase II DUX study evaluated the combination of cetuximab (250 mg m^{-2} per week) and erlotinib (100 mg per day) in 50 patients with chemotherapy-refractory CRC[29]. Treatment was tolerable at these doses, although higher rates of EGFR-related toxicities such as skin rashes and low blood magnesium levels were seen than for either agent alone. The overall response rate was 31%, but no responses were observed in patients with mutant *KRAS*, which was consistent with the known effects of this mutation as a biomarker of resistance to cetuximab monotherapy. In patients with wild-type *KRAS* tumours, the observed response rate was 41%, with

Table 2 | **Clinical trials of antibody and TKI combinations**

Therapeutic candidates*	Trial number‡	ERBB target	Dose	Phase	Cancer type	Outcome	Refs
Cetuximab and gefitinib	NCT00162318	EGFR	100–250 mg m^{-2} cetuximab weekly (escalating dose); 250 mg gefitinib daily	I	NSCLC (refractory to chemotherapy)	MTD established	58
	NCT00820417	EGFR	200–250 mg m^{-2} cetuximab weekly; 100–500 mg gefitinib daily (escalating dose)	I	CRC, head and neck cancer, and NSCLC	MTD established; responses observed in CRC	59
Cetuximab and erlotinib	NCT00408499	EGFR	150–250 mg m^{-2} cetuximab weekly; 100–150 mg erlotinib daily (escalating dose)	I	Various, mainly NSCLC	MTD established	60
	NCT00716456	EGFR	500 mg m^{-2} cetuximab fortnightly; 100 mg erlotinib daily	Ib/II	Lung adenocarcinoma (acquired resistance to erlotinib therapy)	Safety established; no responses observed	61
	NCT00784667 (DUX)	EGFR	250 mg m^{-2} cetuximab weekly; 100 mg erlotinib daily	II	CRC (refractory to chemotherapy)	41% response rate in patients with wild-type *KRAS* tumours	29
Cetuximab and afatinib	NCT01090011	EGFR and ERBB2	500 mg m^{-2} cetuximab fortnightly; 40 mg afatinib daily	Ib/II	NSCLC (acquired resistance to erlotinib therapy)	MTD established; 36% response rate	62
Cetuximab and lapatinib	NCT01184482	EGFR and ERBB2	250 mg m^{-2} cetuximab weekly; 750–1,250 mg lapatinib (escalating dose) daily	I	Various solid tumours	MTD not yet reached	65
Panitumumab and erlotinib plus gemcitabine	NCT00550836	EGFR	4 mg kg^{-1} panitumumab fortnightly; 100 mg erlotinib daily; 1,000 mg m^{-2} gemcitabine weekly	II, randomized	Pancreatic cancer	No improvement in PFS in combination group	66
Trastuzumab and lapatinib	NCT00320385	ERBB2	2 mg kg^{-1} trastuzumab weekly; 1,000 mg lapatinib daily	III, randomized	Trastuzumab-refractory, ERBB2-positive metastatic breast cancer	Improved PFS and overall survival	67
Trastuzumab and lapatinib plus paclitaxel	NCT00553358	ERBB2	2 mg kg^{-1} trastuzumab weekly; 1,000 mg lapatinib daily (biological therapy alone for 6 weeks, then addition of 80 mg m^{-2} paclitaxel weekly for 12 weeks)	III, randomized	ERBB2-positive early breast cancer (neoadjuvant setting)	51% pathological complete response rate for combination group; superior to monotherapy with antibody or TKI (plus paclitaxel)	68

CRC, colorectal cancer; EGFR, epidermal growth factor receptor; MTD, maximum tolerated dose; NSCLC, non-small-cell lung cancer; PFS, progression-free survival; TKI, tyrosine kinase inhibitor. *The antibody is the first listed candidate, and the TKI is the second. Chemotherapeutic agents (paclitaxel and gemcitabine) are included in some trials. ‡ClinicalTrials.gov trial number.

median progression-free survival (PFS) and overall survival times of 5.6 months and 12.9 months, respectively. These results seem promising compared with those for cetuximab monotherapy (response rate of 13%; median PFS of 3.7 months; and median overall survival of 9.5 months)[63]. Further evaluation of this combination in advanced CRC is warranted. Interestingly, these results using cetuximab in combination with a reversible EGFR TKI in CRC seem more encouraging than for their use in NSCLC.

It has been proposed that some of the efficacy in the DUX study may be related to inhibition of other ERBB family members, such as ERBB2, as erlotinib has been shown to have inhibitory effects on this receptor[64]. A similar strategy for CRC involves the combination of the TKI lapatinib, which targets both EGFR and ERBB2, with cetuximab. This combination has been tested in a Phase I study with escalating doses of lapatinib combined with a standard dose of cetuximab[65]. To date, the maximum tolerated dose has not been established.

EGFR inhibitors such as erlotinib have shown limited benefit for advanced pancreatic cancer. A randomized Phase II study compared the combination of the EGFR-specific antibody panitumumab (4 mg kg^{-1} every second week), erlotinib (100 mg per day) and the chemotherapeutic gemcitabine (1,000 mg m^{-2} per week) (a triple combination called PEG) with the combination of gemcitabine plus erlotinib[66]. The study included a lead-in phase to confirm the safety of the triple combination treatment. This study reported a trend towards an overall survival benefit for the PEG combination, but these results should be interpreted with caution, as there was no PFS difference between the study groups. Hence, further randomized studies are required.

Arguably, the strongest data supporting dual targeting of ERBB family members have been observed in breast cancer. A Phase III study of lapatinib and trastuzumab showed superior PFS and overall survival compared with lapatinib in patients with advanced ERBB2-positive cancer who had progressed after having previously been treated with trastuzumab[67]. The benefit in overall survival was observed despite considerable crossover of patients from the lapatinib monotherapy group, which would be expected to lessen the differential efficacy between the two groups. Similar superior efficacy results were also observed in a Phase III study in the neoadjuvant setting (in which patients receive this combination before surgery). The combination of trastuzumab, lapatinib and the chemotherapeutic paclitaxel achieved better pathological complete response rates than lapatinib or trastuzumab plus chemotherapy[68]. This combination is also being evaluated in the adjuvant setting (in which patients receive this combination after surgery).

In summary of the above clinical trials in different tumour types, the combination of cetuximab and reversible EGFR TKIs does not seem to be particularly active against NSCLC. An initial trial with cetuximab and an irreversible TKI displayed encouraging activity, consistent with preclinical data using an irreversible TKI[62]. Dual therapy with cetuximab and erlotinib in CRC showed promising clinical activity[29], but a randomized trial is needed to confirm this. Overall, these early clinical trials suggest that dual EGFR therapy has increased clinical benefit over monotherapy, but the best combination needs to be determined for each tumour type. Dual therapy with trastuzumab and lapatinib, however, shows significant clinical benefit for ERBB2-positive breast cancer[67].

Combination of two or more antibodies. Recently, clinical testing has begun on two EGFR-targeted antibody mixtures (TABLE 3). The first was the Sym004 antibody mixture, which is currently undergoing Phase I/II clinical evaluation in patients with *KRAS*-wild-type refractory or recurrent advanced metastatic CRC who have progressed following EGFR-targeted therapy. Preliminary data from the Phase I component of the trial showed that Sym004 was tolerated well in doses up to 12 mg kg^{-1} with no unexpected toxicities, and preliminary signs of clinical activity were observed[69]. Final results from the Phase I/II study are expected before the end of 2013. An open-label Phase II study of Sym004 in patients with squamous cell carcinoma of the head and neck who failed prior EGFR-targeted therapy is also ongoing. The second EGFR-targeted antibody mixture, MM-151, entered a Phase I trial in early 2013 and consists of three antibodies that target non-overlapping epitopes on EGFR. Thus, the efficacy of targeting EGFR with multiple antibodies against non-overlapping epitopes has yet to be determined.

Trastuzumab was initially approved in 1998 for the treatment of women with ERBB2-positive breast cancers, either as a first-line therapy in combination with paclitaxel chemotherapy or as a single agent for those who have received one or more chemotherapy regimens[70]. Trastuzumab has since also been approved for adjuvant treatment of ERBB2-overexpressing breast cancer, as well as for the treatment of ERBB2-positive metastatic gastric cancers[71,72]. Pertuzumab binds an epitope on ERBB2 that does not overlap with the binding epitope of trastuzumab and, as such, has a mode of action that is complementary to that of trastuzumab. In a Phase II trial, single-agent pertuzumab had limited activity in patients with ERBB2-negative breast cancer. As highlighted above, the combination of trastuzumab and pertuzumab has enhanced preclinical activity in breast and gastric cancer models. Subsequently, the combination was first evaluated in a Phase II clinical trial in patients with advanced ERBB2-positive breast cancer in whom disease progression had occurred during prior trastuzumab-based therapy. The combination of trastuzumab and pertuzumab was well tolerated and showed encouraging results, with an overall response rate of 24.2% and a median PFS of 5.5 months[73]. The encouraging Phase II results prompted a series of clinical trials looking at the activity of the combination in both the metastatic and adjuvant settings.

The CLEOPATRA study investigated the activity of combined trastuzumab and docetaxel chemotherapy with or without pertuzumab in a Phase III randomized study in patients with ERBB2-positive metastatic breast cancer[74]. The median PFS was significantly longer in the pertuzumab group (18.5 months) than in the control group (12.4 months). Although the survival data are not yet complete, the interim analysis of overall survival showed a strong trend in favour of the group treated with trastuzumab in combination with pertuzumab. Importantly, the safety profile was generally similar in both groups, with no increase in left ventricular systolic dysfunction (the most serious side effect seen with trastuzumab and docetaxel) in the antibody combination group. Based on the CLEOPATRA study, in June 2012 the US Food and Drug Administration approved the use of pertuzumab in combination with trastuzumab and docetaxel for the treatment of ERBB2-positive metastatic breast cancer.

Another study for which results have been published is the NEOSPHERE trial. NEOSPHERE investigated the combination of pertuzumab and trastuzumab with or without docetaxel in women with ERBB2-positive breast cancer in the neoadjuvant setting[75]. Results showed that patients receiving pertuzumab, trastuzumab and docetaxel had a significantly improved pathological complete response rate (46%) compared with patients given trastuzumab plus docetaxel (29%). The combination of trastuzumab and pertuzumab

Table 3 | **Clinical trials of antibody combinations**

Therapeutic candidates	Trial number*	ERBB target	Dose	Phase	Cancer type	Outcome	Refs
Sym004 (mixture of two monoclonal antibodies: mAb 992 and mAb 1024)	NCT01117428	EGFR	Up to 12 mg kg^{-1} total dose weekly	I	Advanced solid malignancies	Well tolerated with no unexpected toxicities; preliminary signs of clinical activity	69
			• 9 or 12 mg kg^{-1} total dose weekly • 12 or 18 mg kg^{-1} total dose fortnightly	II	Advanced metastatic CRC (wild-type *KRAS*, refractory or recurrent, progression with prior anti-EGFR-based therapy)	Pending: trial ongoing	None
	NCT01417936	EGFR	12 mg kg^{-1} total dose weekly	II	Squamous cell carcinoma of the head and neck (failed prior anti-EGFR-based therapy)	Pending: trial ongoing	None
MM-151 (mixture of three monoclonal antibodies)	NCT01520389	EGFR	Up to 18 mg kg^{-1} total dose weekly, fortnightly or every third week	I	Advanced solid malignancies	Pending: trial ongoing	None
Trastuzumab and pertuzumab	NCT00301889	ERBB2	2 mg kg^{-1} trastuzumab weekly or 6 mg kg^{-1} trastuzumab every third week plus 420 mg pertuzumab every third week	II	Advanced ERBB2-positive breast cancer (progression with prior trastuzumab-based therapy)	Combination was active and well tolerated	73
Trastuzumab and pertuzumab plus docetaxel	NCT00567190 (CLEOPATRA)	ERBB2	6 mg kg^{-1} trastuzumab plus 420 mg pertuzumab every third week	III, randomized	Metastatic ERBB2-positive breast cancer	Triple combination significantly prolonged PFS compared with trastuzumab plus docetaxel, with no increase in cardiotoxic effects	74
Trastuzumab and pertuzumab with or without docetaxel	NCT00545688 (NEOSPHERE)	ERBB2	6 mg kg^{-1} trastuzumab plus 420 mg pertuzumab every third week	II	ERBB2-positive breast cancer (neoadjuvant setting)	Triple combination significantly improved pathological complete response rate compared with trastuzumab plus docetaxel, without substantial differences in tolerability	75

CRC, colorectal cancer; EGFR, epidermal growth factor receptor; PFS, progression-free survival. *ClinicalTrials.gov trial number.

without chemotherapy eradicated tumours in 17% of patients and showed a favourable safety profile.

Overall, the initial results from clinical trials investigating the activity of combining two antibodies against ERBB2 are very encouraging and confirm preclinical results, suggesting that targeting this receptor with non-overlapping antibodies leads to superior target inhibition. Importantly, the combination seems to be well tolerated, with no increase in cardiac toxicity. If the clinical results with the ERBB2-targeted antibody combination are also seen in the current EGFR clinical trials, this may have a considerable effect on the future development of antibody therapy.

Conclusions and future directions

The preclinical rationale for using two inhibitors of the same receptor, either an antibody and a TKI or two antibodies, has been successfully translated to the clinic for ERBB2-positive breast cancer, and the combination of pertuzumab, trastuzumab and docetaxel is now approved. Dual therapy using a specific antibody in combination with TKIs against EGFR-positive tumours has generated results that are more ambiguous. However, several encouraging clinical trials suggest that certain combinations have enhanced activity in particular types of cancer; confirmation of these findings awaits additional randomized trials. Clinical data from trials of dual EGFR-targeted antibodies are eagerly awaited, given the overwhelming preclinical data that support this approach. Until recently, there has been no driving hypothesis for using two TKIs in combination. However, recent data on glioma[48] suggest that using TKIs against both the open and the closed conformations of the EGFR kinase domain might have broader antitumour activity than single-agent TKIs.

Many of the preclinical studies using two EGFR inhibitors in combination were published before the effect of *EGFR* mutations on the therapeutic response was well described, particularly for NSCLC. Therefore, our knowledge of the influence of *EGFR* genotype on the effectiveness of these combinations has not been extensively examined and needs further investigation. Likewise, the influence of *KRAS* mutational status has not been effectively explored in preclinical models, although the combination of cetuximab and erlotinib failed to produce a clinical response in any patients with *KRAS*-mutant colon cancer[29].

The most obvious difference between dual therapy with a TKI and an antibody versus a combination of antibodies is that

TKI dual therapy increases the amount of cell-surface EGFR but that antibody combination therapy dramatically reduces it. Somewhat paradoxically, both strategies seem to enhance ADCC activity, albeit by different mechanisms; therefore, it is unlikely that either approach would have an advantage in this regard. One clear difference between the two approaches is that only the antibody combinations have the potential to inhibit the non-kinase activities of EGFR, as these combinations remove the receptor from the cell surface. Given that studies on the importance of non-kinase functions of EGFR in cancer remain in their infancy, the relevance of this with respect to a possible advantage for dual antibody therapy remains unknown. However, if further studies indicate that the non-kinase functions of EGFR are important, then dual antibody approaches may be a more effective clinical strategy than a TKI and an antibody. More generally, whether either approach is more effective for different types of cancer can be determined only by ongoing clinical trials.

There is considerable scope for further advances based on this large body of work in relation to dual inhibition, which has moved from discovery to clinical practice in less than a decade. One area of particularly intense research is the development of novel antibody constructs that can bind multiple sites on EGFR. For example, a novel tri-epitopic antibody fusion that combines three different EGFR epitopes in one molecule has been shown to inhibit cetuximab-resistant *KRAS*-mutant tumours in colon cancer xenograft models[76]. Another antibody of considerable interest is MEHD7945A, which is a conventional antibody that can bind both EGFR and ERBB3 simultaneously[77]. This antibody can inhibit both EGFR and ERBB3 signalling and has broader antitumour activity against a range of tumours than single agents specific for either receptor. Given the recent observation that ERBB3 might have low levels of kinase activity[5], ERBB3 may cause *de novo* resistance to EGFR and/or ERBB2 inhibitors, thus its direct inhibition might enhance the activity of these inhibitors. More generally, targeting strategies that minimize heterodimerization and crosstalk between ERBB family members should be the focus of additional development strategies, as the success of the pertuzumab and trastuzumab combination shows that preventing these activities improves clinical outcomes.

Overall, these studies demonstrate that the best inhibition of tumour growth is obtained by minimizing signalling from the targeted receptor. Clearly, single agents are not sufficient to completely block the signalling of ERBB family members or the crosstalk between them. The lessons learned from this group of receptors should also apply to other RTKs that are activated in cancer. Indeed, preclinical data have validated this approach in several RTK systems, including the combination of two antibodies that inhibit insulin-like growth factor 1 receptor[78]. The strategy of dual inhibition should continue to find additional clinical uses, leading to improvements in patient outcomes.

Terrance G. Johns is at the Monash Institute of Medical Research, 27–31 Wright Street, Clayton, Victoria 3168, Australia.

Mikkel W. Pedersen is at Symphogen, Elektrovej Building 375, 2800 Lyngby, Denmark.

Niall Tebbutt is at the Ludwig Oncology Unit, Austin Health, Studley Road, Heidelberg, Victoria 3084, Australia.

Correspondence to T.G.J.
e-mail: Terry.Johns@monash.edu

doi:10.1038/nrc3559
Published online 16 August 2013

1. Casalini, P., Iorio, M. V., Galmozzi, E. & Menard, S. Role of HER receptors family in development and differentiation. *J. Cell. Physiol.* **200**, 343–350 (2004).
2. Prenzel, N., Fischer, O. M., Streit, S., Hart, S. & Ullrich, A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr. Relat. Cancer* **8**, 11–31 (2001).
3. Roskoski, R. Jr. The ErbB/HER receptor protein-tyrosine kinases and cancer. *Biochem. Biophys. Res. Commun.* **319**, 1–11 (2004).
4. Hynes, N. E., Horsch, K., Olayioye, M. A. & Badache, A. The ErbB receptor tyrosine family as signal integrators. *Endocr. Relat. Cancer* **8**, 151–159 (2001).
5. Shi, F., Telesco, S. E., Liu, Y., Radhakrishnan, R. & Lemmon, M. A. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. *Proc. Natl Acad. Sci. USA* **107**, 7692–7697 (2010).
6. Zhang, Q., Park, E., Kani, K. & Landgraf, R. Functional isolation of activated and unilaterally phosphorylated heterodimers of ERBB2 and ERBB3 as scaffolds in ligand-dependent signaling. *Proc. Natl Acad. Sci. USA* **109**, 13237–13242 (2012).
7. Yarden, Y. & Pines, G. The ERBB network: at last, cancer therapy meets systems biology. *Nature Rev. Cancer* **12**, 553–563 (2012).
8. Hynes, N. E. & MacDonald, G. ErbB receptors and signaling pathways in cancer. *Curr. Opin. Cell Biol.* **21**, 177–184 (2009).
9. Sundvall, M. *et al.* Role of ErbB4 in breast cancer. *J. Mammary Gland Biol. Neoplasia* **13**, 259–268 (2008).
10. Prickett, T. D. *et al.* Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in ERBB4. *Nature Genet.* **41**, 1127–1132 (2009).
11. Albanell, J. & Gascon, P. Small molecules with EGFR-TK inhibitor activity. *Curr. Drug Targets* **6**, 259–274 (2005).
12. Mendelsohn, J. & Baselga, J. Epidermal growth factor receptor targeting in cancer. *Semin. Oncol.* **33**, 369–385 (2006).
13. Bria, E. *et al.* Outcome of advanced NSCLC patients harboring sensitizing EGFR mutations randomized to EGFR tyrosine kinase inhibitors or chemotherapy as first-line treatment: a meta-analysis. *Ann. Oncol.* **22**, 2277–2285 (2011).
14. Wheeler, D. L., Dunn, E. F. & Harari, P. M. Understanding resistance to EGFR inhibitors — impact on future treatment strategies. *Nature Rev. Clin. Oncol.* **7**, 493–507 (2010).
15. Siena, S., Sartore-Bianchi, A., Di Nicolantonio, F., Balfour, J. & Bardelli, A. Biomarkers predicting clinical outcome of epidermal growth factor receptor-targeted therapy in metastatic colorectal cancer. *J. Natl Cancer Inst.* **101**, 1308–1324 (2009).
16. Schmitz, K. R. & Ferguson, K. M. Interaction of antibodies with ErbB receptor extracellular regions. *Exp. Cell Res.* **315**, 659–670 (2009).
17. Bos, M. *et al.* PD153035, a tyrosine kinase inhibitor, prevents epidermal growth factor receptor activation and inhibits growth of cancer cells in a receptor number-dependent manner. *Clin. Cancer Res.* **3**, 2099–2106 (1997).
18. Johns, T. G. *et al.* Antitumor efficacy of cytotoxic drugs and the monoclonal antibody 806 is enhanced by the EGF receptor inhibitor AG1478. *Proc. Natl Acad. Sci. USA* **100**, 15871–15876 (2003).
19. Gan, H. K. *et al.* The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor AG1478 increases the formation of inactive untethered EGFR dimers. Implications for combination therapy with monoclonal antibody 806. *J. Biol. Chem.* **282**, 2840–2850 (2007).
20. Perera, R. M. *et al.* Treatment of human tumor xenografts with monoclonal antibody 806 in combination with a prototypical epidermal growth factor receptor-specific antibody generates enhanced antitumor activity. *Clin. Cancer Res.* **11**, 6390–6399 (2005).
21. Matar, P. *et al.* Combined epidermal growth factor receptor targeting with the tyrosine kinase inhibitor gefitinib (ZD1839) and the monoclonal antibody cetuximab (IMC-C225): superiority over single-agent receptor targeting. *Clin. Cancer Res.* **10**, 6487–6501 (2004).
22. Huang, S., Armstrong, E. A., Benavente, S., Chinnaiyan, P. & Harari, P. M. Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR): combining anti-EGFR antibody with tyrosine kinase inhibitor. *Cancer Res.* **64**, 5355–5362 (2004).
23. Jimeno, A. *et al.* Epidermal growth factor receptor dynamics influences response to epidermal growth factor receptor targeted agents. *Cancer Res.* **65**, 3003–3010 (2005).
24. Scaltriti, M. *et al.* Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. *Oncogene* **28**, 803–814 (2009).
25. Rimawi, M. F. *et al.* Reduced dose and intermittent treatment with lapatinib and trastuzumab for potent blockade of the HER pathway in HER2/neu-overexpressing breast tumor xenografts. *Clin. Cancer Res.* **17**, 1351–1361 (2011).
26. Wainberg, Z. A. *et al.* Lapatinib, a dual EGFR and HER2 kinase inhibitor, selectively inhibits HER2-amplified human gastric cancer cells and is synergistic with trastuzumab in vitro and in vivo. *Clin. Cancer Res.* **16**, 1509–1519 (2010).
27. Scheuer, W. *et al.* Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models. *Cancer Res.* **69**, 9330–9336 (2009).
28. Yamashita-Kashima, Y. *et al.* Pertuzumab in combination with trastuzumab shows significantly enhanced antitumor activity in HER2-positive human gastric cancer xenograft models. *Clin. Cancer Res.* **17**, 5060–5070 (2011).
29. Weickhardt, A. J. *et al.* Dual targeting of the epidermal growth factor receptor using the combination of cetuximab and erlotinib: preclinical evaluation and results of the phase II DUX study in chemotherapy-refractory, advanced colorectal cancer. *J. Clin. Oncol.* **30**, 1505–1512 (2012).
30. Regales, L. *et al.* Dual targeting of EGFR can overcome a major drug resistance mutation in mouse models of EGFR mutant lung cancer. *J. Clin. Invest.* **119**, 3000–3010 (2009).
31. Huang, P. H. *et al.* Quantitative analysis of EGFRvIII cellular signaling networks reveals a combinatorial therapeutic strategy for glioblastoma. *Proc. Natl Acad. Sci. USA* **104**, 12867–12872 (2007).
32. Alvarado, D., Klein, D. E. & Lemmon, M. A. ErbB2 resembles an autoinhibited invertebrate epidermal growth factor receptor. *Nature* **461**, 287–291 (2009).
33. Huang, S. *et al.* Dual targeting of EGFR and HER3 with MEHD7945A overcomes acquired resistance to EGFR inhibitors and radiation. *Cancer Res.* **73**, 824–833 (2013).

34. Yonesaka, K. *et al.* Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. *Sci. Transl. Med.* **3**, 99ra86 (2011).
35. Garrett, J. T., Sutton, C. R., Kuba, M. G., Cook, R. S. & Arteaga, C. L. Dual blockade of HER2 in HER2-overexpressing tumor cells does not completely eliminate HER3 function. *Clin. Cancer Res.* **19**, 610–619 (2013).
36. Cavazzoni, A. *et al.* Combined use of anti-ErbB monoclonal antibodies and erlotinib enhances antibody-dependent cellular cytotoxicity of wild-type erlotinib-sensitive NSCLC cell lines. *Mol. Cancer* **11**, 91 (2012).
37. Bibeau, F. *et al.* Impact of FcγRIIa-FcγRIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. *J. Clin. Oncol.* **27**, 1122–1129 (2009).
38. Varchetta, S. *et al.* Elements related to heterogeneity of antibody-dependent cell cytotoxicity in patients under trastuzumab therapy for primary operable breast cancer overexpressing Her2. *Cancer Res.* **67**, 11991–11999 (2007).
39. Wang, Q. *et al.* Concomitant targeting of tumor cells and induction of T-cell response synergizes to effectively inhibit trastuzumab-resistant breast cancer. *Cancer Res.* **72**, 4417–4428 (2012).
40. Stagg, J. *et al.* Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. *Proc. Natl Acad. Sci. USA* **108**, 7142–7147 (2011).
41. Spangler, J. B. *et al.* Combination antibody treatment down-regulates epidermal growth factor receptor by inhibiting endosomal recycling. *Proc. Natl Acad. Sci. USA* **107**, 13252–13257 (2010).
42. Schmidt, M. H., Furnari, F. B., Cavenee, W. K. & Bogler, O. Epidermal growth factor receptor signaling intensity determines intracellular protein interactions, ubiquitination, and internalization. *Proc. Natl Acad. Sci. USA* **100**, 6505–6510 (2003).
43. Pedersen, M. W. *et al.* Sym004: a novel synergistic anti-epidermal growth factor receptor antibody mixture with superior anticancer efficacy. *Cancer Res.* **70**, 588–597 (2010).
44. Zhu, H., Cao, X., Ali-Osman, F., Keir, S. & Lo, H. W. EGFR and EGFRvIII interact with PUMA to inhibit mitochondrial translocalization of PUMA and PUMA-mediated apoptosis independent of EGFR kinase activity. *Cancer Lett.* **294**, 101–110 (2010).
45. Weihua, Z. *et al.* Survival of cancer cells is maintained by EGFR independent of its kinase activity. *Cancer Cell* **13**, 385–393 (2008).
46. Xu, S. & Weihua, Z. Loss of EGFR induced autophagy sensitizes hormone refractory prostate cancer cells to adriamycin. *Prostate* **71**, 1216–1224 (2011).
47. Taylor, T. E., Furnari, F. B. & Cavenee, W. K. Targeting EGFR for treatment of glioblastoma: molecular basis to overcome resistance. *Curr. Cancer Drug Targets* **12**, 197–209 (2012).
48. Vivanco, I. *et al.* Differential sensitivity of glioma- versus lung cancer-specific EGFR mutations to EGFR kinase inhibitors. *Cancer Discov.* **2**, 458–471 (2012).
49. Hegi, M. E. *et al.* Pathway analysis of glioblastoma tissue after preoperative treatment with the EGFR tyrosine kinase inhibitor gefitinib — a phase II trial. *Mol. Cancer Ther.* **10**, 1102–1112 (2011).
50. Gan, H. K., Kaye, A. H. & Luwor, R. B. The EGFRvIII variant in glioblastoma multiforme. *J. Clin. Neurosci.* **16**, 748–754 (2009).
51. Heimberger, A. B. *et al.* Brain tumors in mice are susceptible to blockade of epidermal growth factor receptor (EGFR) with the oral, specific, EGFR-tyrosine kinase inhibitor ZD1839 (iressa). *Clin. Cancer Res.* **8**, 3496–3502 (2002).
52. Oxnard, G. R. *et al.* New strategies in overcoming acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in lung cancer. *Clin. Cancer Res.* **17**, 5530–5537 (2011).
53. Brand, T. M., Iida, M., Li, C. & Wheeler, D. L. The nuclear epidermal growth factor receptor signaling network and its role in cancer. *Discov. Med.* **12**, 419–432 (2011).
54. Li, C., Iida, M., Dunn, E. F., Ghia, A. J. & Wheeler, D. L. Nuclear EGFR contributes to acquired resistance to cetuximab. *Oncogene* **28**, 3801–3813 (2009).
55. Huang, W. C. *et al.* Nuclear translocation of epidermal growth factor receptor by Akt-dependent phosphorylation enhances breast cancer-resistant protein expression in gefitinib-resistant cells. *J. Biol. Chem.* **286**, 20558–20568 (2011).
56. Cvrljevic, A. N. *et al.* Activation of Src induces mitochondrial localisation of de2-7EGFR (EGFRvIII) in glioma cells: implications for glucose metabolism. *J. Cell Sci.* **124**, 2938–2950 (2011).
57. Demory, M. L. *et al.* Epidermal growth factor receptor translocation to the mitochondria: regulation and effect. *J. Biol. Chem.* **284**, 36592–36604 (2009).
58. Ramalingam, S. *et al.* Dual inhibition of the epidermal growth factor receptor with cetuximab, an IgG1 monoclonal antibody, and gefitinib, a tyrosine kinase inhibitor, in patients with refractory non-small cell lung cancer (NSCLC): a phase I study. *J. Thorac. Oncol.* **3**, 258–264 (2008).
59. Baselga, J. *et al.* A phase I pharmacokinetic (PK) and molecular pharmacodynamic (PD) study of the combination of two anti-EGFR therapies, the monoclonal antibody (MAb) cetuximab (C) and the tyrosine kinase inhibitor (TKI) gefitinib (G), in patients (pts) with advanced colorectal (CRC), head and neck (HNC) and non-small cell lung cancer (NSCLC). *J. Clin. Oncol. Abstr.* **24**, 3006 (2006).
60. Sangha, R. *et al.* Dual epidermal growth factor receptor (EGFR) inhibition: phase I study combining cetuximab (C225) and erlotinib (E) in advanced solid tumors. *J. Clin. Oncol. Abstr.* **27**, 3552 (2009).
61. Janjigian, Y. Y. *et al.* Phase I/II trial of cetuximab and erlotinib in patients with lung adenocarcinoma and acquired resistance to erlotinib. *Clin. Cancer Res.* **17**, 2521–2527 (2011).
62. Janjigian, Y. Y. *et al.* Activity and tolerability of afatinib (BIBW 2992) and cetuximab in NSCLC patients with acquired resistance to erlotinib or gefitinib. *J. Clin. Oncol. Abstr.* **29**, 7525 (2011).
63. Karapetis, C. S. *et al.* K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N. Engl. J. Med.* **359**, 1757–1765 (2008).
64. Schaefer, G., Shao, L., Totpal, K. & Akita, R. W. Erlotinib directly inhibits HER2 kinase activation and downstream signaling events in intact cells lacking epidermal growth factor receptor expression. *Cancer Res.* **67**, 1228–1238 (2007).
65. Deeken, J. F. *et al.* A phase I study of lapatinib (LPT) and cetuximab (CTX) in patients with CTX-sensitive solid tumors. *J. Clin. Oncol. Abstr.* **30**, 2590 (2012).
66. Kim, G. P. *et al.* Randomized phase II trial of panitumumab, erlotinib, and gemcitabine (PGE) versus erlotinib-gemcitabine (GE) in patients with untreated, metastatic pancreatic adenocarcinoma. *J. Clin. Oncol. Abstr.* **29**, 4030 (2011).
67. Blackwell, K. L. *et al.* Randomized study of Lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. *J. Clin. Oncol.* **28**, 1124–1130 (2010).
68. Baselga, J. *et al.* Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. *Lancet* **379**, 633–640 (2012).
69. Dienstmann, R. *et al.* Phase I trial of the first-in-class EGFR antibody mixture, Sym004, in patients with advanced solid tumors. *J. Clin. Oncol. Abstr.* **29**, 3089 (2011).
70. Cobleigh, M. A. *et al.* Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J. Clin. Oncol.* **17**, 2639–2648 (1999).
71. Gianni, L. *et al.* Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: a 4-year follow-up of a randomised controlled trial. *Lancet Oncol.* **12**, 236–244 (2011).
72. Smith, I. *et al.* 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet* **369**, 29–36 (2007).
73. Baselga, J. *et al.* Phase II trial of pertuzumab and trastuzumab in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer that progressed during prior trastuzumab therapy. *J. Clin. Oncol.* **28**, 1138–1144 (2010).
74. Baselga, J. *et al.* Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N. Engl. J. Med.* **366**, 109–119 (2012).
75. Gianni, L. *et al.* Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. *Lancet Oncol.* **13**, 25–32 (2012).
76. Spangler, J. B., Manzari, M. T., Rosalia, E. K., Chen, T. F. & Wittrup, K. D. Triepitopic antibody fusions inhibit cetuximab-resistant BRAF and KRAS mutant tumors via EGFR signal repression. *J. Mol. Biol.* **422**, 532–544 (2012).
77. Schaefer, G. *et al.* A two-in-one antibody against HER3 and EGFR has superior inhibitory activity compared with monospecific antibodies. *Cancer Cell* **20**, 472–486 (2011).
78. Dong, J. *et al.* Combination of two insulin-like growth factor-I receptor inhibitory antibodies targeting distinct epitopes leads to an enhanced antitumor response. *Mol. Cancer Ther.* **9**, 2593–2604 (2010).
79. Vincenzi, B., Zoccoli, A., Pantano, F., Venditti, O. & Galluzzo, S. Cetuximab: from bench to bedside. *Curr. Cancer Drug Targets* **10**, 80–95 (2010).
80. Schneider-Merck, T. *et al.* Human IgG2 antibodies against epidermal growth factor receptor effectively trigger antibody-dependent cellular cytotoxicity but, in contrast to IgG1, only by cells of myeloid lineage. *J. Immunol.* **184**, 512–520 (2010).
81. Garrett, T. P. *et al.* Antibodies specifically targeting a locally misfolded region of tumor associated EGFR. *Proc. Natl Acad. Sci. USA* **106**, 5082–5087 (2009).
82. Johns, T. G. *et al.* Identification of the epitope for the epidermal growth factor receptor-specific monoclonal antibody 806 reveals that it preferentially recognizes an untethered form of the receptor. *J. Biol. Chem.* **279**, 30375–30384 (2004).
83. Yoshida, T., Zhang, G. & Haura, E. B. Targeting epidermal growth factor receptor: central signaling kinase in lung cancer. *Biochem. Pharmacol.* **80**, 613–623 (2010).
84. Linardou, H., Dahabreh, I. J., Bafaloukos, D., Kosmidis, P. & Murray, S. Somatic EGFR mutations and efficacy of tyrosine kinase inhibitors in NSCLC. *Nature Rev. Clin. Oncol.* **6**, 352–366 (2009).
85. Boschelli, D. H. 4-anilino-3-quinolinecarbonitriles: an emerging class of kinase inhibitors. *Curr. Top. Med. Chem.* **2**, 1051–1063 (2002).
86. Hudis, C. A. Trastuzumab--mechanism of action and use in clinical practice. *N. Engl. J. Med.* **357**, 39–51 (2007).
87. Reid, A., Vidal, L., Shaw, H. & de Bono, J. Dual inhibition of ErbB1 (EGFR/HER1) and ErbB2 (HER2/neu). *Eur. J. Cancer* **43**, 481–489 (2007).
88. Xia, W. *et al.* Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* **21**, 6255–6263 (2002).
89. Li, D. *et al.* BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. *Oncogene* **27**, 4702–4711 (2008).
90. Spicer, J. F. & Rudman, S. M. EGFR inhibitors in non-small cell lung cancer (NSCLC): the emerging role of the dual irreversible EGFR/HER2 inhibitor BIBW 2992. *Target Oncol.* **5**, 245–255 (2010).

Acknowledgements

T.G.J.'s work on ERBB family inhibitors is supported by funding from the National Health and Medical Research Council of Australia (grant numbers 1012020 and 1028552) and the Victorian Government's Operational Infrastructure Support Program. T.G.J. is a recipient of a Clinical Fellowship from the Victorian Cancer Agency. The authors thank D. Dadley-Moore for help with editing the manuscript.

Competing interests statement

The authors declare competing financial interests: see Web version for details.