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Targeting erbB receptors

Zheng Cai^a, Hongtao Zhang^a, Jing Liu^b, Alan Berezov^c, Ramachandran Murali^d, Qiang Wang^e, and Mark I. Greene^{a,*}

^aDepartment of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104-6082, USA

^bSchool of Life Sciences, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230027, China

^cDepartment of Biomedical Sciences, Cedars-Sinai Medical Center, S122A Steven Spielberg Building, 8725 Alden Dr., Los Angeles, CA 90048, USA

^dDepartment of Biomedical Sciences, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Davis Building, # 4092, Los Angeles, CA 90048, USA

^eWomen's Cancer Research Institute at the Samuel Oschin Comprehensive Cancer Institute, Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

Abstract

Our work is concerned with the origins and therapy of human cancers. Members of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, also known as erbB or HER receptors, are over expressed and/or activated in many types of human tumors and represent important therapeutic targets in cancer therapy. Studies from our laboratory identified targeted therapy as a way to treat cancer. Rational therapeutics targeting and disabling erbB receptors have been developed to reverse the malignant properties of tumors. Reversal of the malignant phenotype, best seen with disabling the HER2 receptors using monoclonal antibodies is a distinct process from that seen with blocking of ligand binding to cognate receptors as has been done for EGFR receptors. Here we review the mechanisms of action deduced from a number of approaches developed in our laboratory and elsewhere, including monoclonal antibodies, peptide mimetics, recombinant proteins and small molecules. The biochemical and biological principles which have been uncovered during these studies of disabling HER2 homomeric or HER2-EGFR heteromeric receptors will help the development of novel and more efficient therapeutics targeting erbB family receptors.

Keywords

erbB receptors; Targeted therapy; Monoclonal antibody; Peptide mimetic; Recombinant protein

*Corresponding author at: University of Pennsylvania School of Medicine, Department of Pathology and Laboratory Medicine, 252 John Morgan Building, 36th Street, Hamilton Walk, Philadelphia, PA 19104-6082, USA. Tel.: +1 215 898 2868; fax: +1 215 898 2401. greenem@reo.med.upenn.edu (M.I. Greene).

1. Introduction

The epidermal growth factor receptor (EGFR) family of tyrosine kinases, also known as erbB or HER receptors, are over expressed and/or activated in many types of human tumors. There are four members in the erbB family: EGFR, HER2 (also known as p185^{her2/neu} and Neu), HER3 (erbB3) and HER4 (erbB4). Activation of erbB receptors leads not only to increased cell proliferation, but also to resistance to growth-inhibitory cytokines. Moreover the activated erbB receptors lead to a transformed phenotype which is associated with the expression of selective immune suppressive and proangiogenic cytokines and chemokines. This tumor-host interaction creates an environment that favors tumor progression [1,2]. All members of the erbB family share structural similarities, which include an extracellular ligand binding domain, a transmembrane domain, and an intracellular kinase domain.

The erbB family of tyrosine kinases have emerged as important therapeutic targets in oncology. Receptor-directed therapy represents a powerful cancer management strategy. Numerous antibodies, recombinant proteins, peptide mimetics and small molecules have been developed for targeting EGF receptors. Some of the antibodies (cetuximab, panitumumab, and trastuzumab) and small molecules (gefitinib, erlotinib, and lapatinib) have been approved clinically and are already benefiting patients.

Although the results are encouraging, there is still a need for the development of novel and more efficient therapies, because many patients are either not sensitive to current drugs/antibodies or develop resistance after a few months of treatment. The mechanisms by which therapeutics exert their activity on malignant properties are incompletely defined and the multiple pathways by which resistance to targeted therapy arises are also poorly understood.

Our laboratory initiated the rational therapeutic targeting and disabling of erbB receptors to reverse the malignant properties of tumors. Reversal of phenotype is very distinct from blocking of ligand binding to cognate receptors as has been done for EGFR receptors. We have developed a number of approaches including monoclonal antibodies, peptide mimetics, recombinant proteins and small molecules. Here we review the results obtained in our laboratory and elsewhere for erbB targeted therapy mediated by various therapeutic modalities.

2. Antibodies

For many years, monoclonal antibodies were considered primarily useful for molecular diagnostics. However, monoclonal antibodies have been developed for anti-cancer treatment, and they have emerged as a class of biologic cancer therapeutics. We generated a panel of monoclonal antibodies against the rat p185^{neu} receptor, including 7.16.4, 7.21.2 and A11 [3]. These antibodies have high affinities for the oncogenic rodent p185^{neu} receptor. From staining of human malignant tissues, we observed that 7.16.4 also interacts with p185^{her2/neu} on human breast adenocarcinoma cells [4] and other human tissues (Paco Real, Mark Greene, 1986, unpublished data). The 7.16.4 can effectively inhibit anchorage dependent cell growth under most serum conditions although this is clearly dependent on the characteristics of the fetal calf serum used. Many of these monoclonals including the

prototypic 7.16.4 can also limit anchorage independent growth and reverse the phenotype of cells transformed by either mutant p185^{neu} or by over expressed p185^{her2/neu} [3].

mAb 7.16.4 is able to bind to human tumor tissues in a manner comparable to the binding and staining of rhuMAB4D5. 7.16.4 can readily displace rhuMAB4D5 (a humanized anti-p185^{her2/neu} antibody developed several years later and independently by scientists at Genentech) from its interaction with p185^{her2/neu} in p185^{her2/neu} expressing T6–17 cells [4]. More importantly, rhuMAB4D5 and 7.16.4 bind to the same or an overlapping epitope on the ectodomain of p185^{her2/neu}. RhuMAB4D5 was only slightly more effective (IC50, 1 µg/ml) at reversing phenotype than the 7.16.4 monoclonal antibody (IC50, 10 µg/ml). These studies indicated that both 7.16.4 and 4D5 interact with the same epitope and have similar biological activities and mediate their activities by the same mechanisms. Mechanisms of phenotype reversal include creation of ineffective tetramers which lack kinase activity (Fig. 1).

A chimeric anti-p185^{her2/neu} antibody chA21 that specifically inhibits the growth of p185^{her2/neu}-over expressing cancer cells *in vitro* and *in vivo* has also been developed ([5] and Huihao Zhou et al., unpublished data). As suggested by the crystal structure of the single-chain Fv (ScFv) fragment of chA21 in complex with an N-terminal fragment of the p185^{her2/neu} ectodomain, chA21 binds to an epitope located diametrically opposite to the putative p185^{her2/neu} dimerization interface, indicating that chA21 does not directly block the dimerization of p185^{her2/neu} species.

The chA21 epitope is distinct from those of the other anti-p185^{her2/neu} therapeutic antibodies including Trastuzumab(4D5) and Pertuzumab(2C4). Binding of the bivalent monoclonal antibody chA21 to its distinct epitope also leads to internalization and downregulation of p185^{her2/neu} in SK-BR-3 cells (Huihao Zhou et al., unpublished data).

We previously demonstrated that therapeutic monoclonal antibodies can promote EGFR or p185^{her2/neu}-EGFR to form tetramers. EGF-induced phosphorylation in the tetramers was significantly lower than that of the dimers [6]. The impaired receptor tetramers were observed after even a brief 15-min treatment with a mixture of mAbs, and these tetramers had significantly diminished kinase activity. Our study also demonstrated that formation of the impaired tetrameric receptor forms was a physiologically relevant process largely independent of the receptor density. We also showed that antibodies can rapidly shift the equilibrium from active dimeric to an impaired tetrameric receptor complex state. This mechanism may present an important component for the antibodies' antitumor activity (Fig. 1A) and indicates a rapid time frame for reversal of the malignant phenotype.

We also sought to understand the mechanism by which chA21 functions because of its unique interaction epitope. We found that monovalent ScFv of chA21 was not able to induce p185^{her2/neu} down-regulation, whereas bivalent chA21 could produce that effect. Furthermore, the chA21-mediated p185^{her2/neu} down-regulation is dose-dependent in a bell-shaped curve reflecting the stoichiometry of the process. These data lead to a cross-linking model, in which chA21 interacts with two p185^{her2/neu} molecules on separate homo- or hetero dimers, resulting in cross-linking of these dimers to form a large complex in the cell membrane for internalization and degradation (Fig. 1B).

This mechanism is also reminiscent of our previous work documenting that internalization of receptors can be enhanced by antibodies that promote active receptor dimers to associate into inactive tetramers.

3. Peptide mimetics

We developed two additional ectodomain targeted approaches for disabling erbB receptor signaling using mimetic peptides. The first approach was focused on mimicking the effects of anti-erbB receptor MABs, whereas the second approach targets receptor dimerization interfaces. Both strategies have proven efficient in the inhibition of erbB receptor functions in both *in vitro* and *in vivo* studies.

3.1. Antibody mimetics, novel antibody surrogates created by using the deconstructed heavy chain CDR3, typified by AHNP

There are appreciated limitations associated with the systemic use of intact antibody molecules as therapeutic or diagnostic entities. They may engender a host immune response, possess little ability to penetrate into tumors and practically are associated with a high production cost [7,8]. Attempts to reduce the size of antibodies resulted in the development of smaller versions of antibodies that retain specificity [single-chain Fvs, Fabs, Fab(2)s, minibodies, domain-deleted antibodies] and have increased penetration into solid tumors [9]. A promising alternative approach to overcome the limitations of high-molecular-weight therapeutics was to design mimetic peptides derived from the antigen-binding site of antibodies.

Our approach was to deconstruct the antibody binding site. Based on our crystallographic studies of protein antigen complexes we recognized that the CDR3 of the heavy chain surrounded by aromatics created a highly energetic surface for antigen binding [10]. Through studies of the deconstructed antigen binding surface of a monoclonal antibody we established a general principle that a single CDR3 loop from the heavy chain of the antibody is sufficient to mimic its parental monoclonal antibody function [11–16]. The CDR peptide is much smaller than the antibody but retains binding affinity to the antigen as well as the biological activity of the antibody. Our studies of the CDR mimetics demonstrated that the constrained CDRs could mediate binding in a context independent manner and formed the theoretical basis for the technique of CDR grafting [11,12,17]. We also extended this notion to create a synthetic CDR peptidomimetic species that was biologically active [15].

Based on the shared structural properties of the third CDR of the heavy chain of the 7.16.4 and rhuMAB4D5 antibodies, we developed a 1.5 kDa anti-HER2 peptidomimetic named AHNP (anti her2/neu peptide). The structure of AHNP was determined by solution NMR (Fig. 2) and demonstrated a single conformation. It also revealed structural features distinct from molecular modeling. These features were then incorporated into newly designed peptidomimetics. As shown by both *in vitro* and *in vivo* studies, AHNP exhibits the activity to disable p185^{her2/neu} tyrosine kinases to the extent comparable to that of the monoclonal antibodies [17].

AHNP has been shown to bind to p185^{her2/neu} with submicromolar affinity. It inhibited proliferation of p185^{her2/neu}-expressing tumor cells. The peptide mimetic also showed good activity in both anchorage-independent colony growth assays and most importantly in the growth of p185^{her2/neu}-expressing tumors in athymic mice. In addition, AHNP sensitized the tumor cells to apoptosis when it was used in conjunction with ionizing radiation or chemotherapeutic agents and was as effective as Herceptin in reducing tumor size and more effective than Herceptin in its ability to inhibit proliferation.

However while the intact monoclonal antibodies actively down regulate p185^{her2/neu} from the cell surface, AHNP has no effect on receptor down regulation, indicating that the small molecule disables the p185^{her2/neu} complex principally by promoting misaligned monomers and limiting the monomers ability to create a stable dimer with an activated kinase. Our data showed that AHNP indicating a shared binding site for the peptide mimetic and the parental antibody.

To further develop AHNP as an antitumor agent useful for preclinical trials and as a radiopharmaceutical to be used for tumor imaging, a number of derivatives of AHNP have been designed. These second generation species have important advantages over AHNP in terms of their binding properties, specificity, and solubility [18]. Structure-function relationships have been studied using surface plasmon resonance technology. Some of the AHNP analogues have improved binding properties, solubility, and cytotoxic activity relative to AHNP. Residues in the exocyclic region of AHNP are essential for high-affinity binding. Kinetic and equilibrium analysis of peptide receptor binding for various AHNP analogues revealed a strong correlation between peptide binding characteristics and their biological activity. We also showed that the dissociation rate constant is a better indicator than receptor binding affinities for predicting the biological activity of AHNP analogues.

The antitumor effects of AHNP can be enhanced when combined with chemotherapeutic drugs. For example, a dimeric AHNP peptide (AHNP bivalent) was designed to conjugate with Taxol to deliver the drug into tumor cells through p185^{Her2/neu} internalization [19]. The Taxol conjugated AHNP bivalent prodrug binds to p185^{Her2/neu}, and induces receptor internalization and down regulation. Free Taxol is subsequently released inside the targeted cell and results in additional cytotoxicity. This strategy can be generalized to selectively target cancer cells by directing cytotoxic drugs into the tumor-marker expressing cells, through receptor-mediated internalization.

Our efforts revealed the possibility of mimicking the well-documented antibody effects and its applications in tumor therapy using much smaller antibody-based cyclic peptides with significant therapeutic advantages.

To improve AHNP efficacy, we engineered a fusion protein containing AHNP and a nonimmunoglobulin protein, streptavidin [20]. The recombinant protein, AHNP-SA (ASA) bound to p185^{her2/neu} with a high affinity (Biacore: 8.8 nM), inhibited the proliferation of p185^{her2/neu}-overexpressing cells, and reduced tumor growth induced by p185^{her2/neu} transformed cells. Compared with AHNP, ASA showed less activity in an anchorage-independent assay using polyHEMA but more activity in an *in vivo* tumor growth assay.

We expected ASA to possess a serum half-life close to that of SA (12 h) [21] since the newly developed ASA species has a similar core structure and molecular weight to that of SA. The AHNP molecule's serum half-life is about 2–3 h. Our studies revealed that the bacterially produced tetrameric ASA can be used as an antibody-surrogate molecule. ASA can be also used as a diagnostic tool to detect p185^{her2/neu} in early breast cancer developments using FACTT, an ultra-sensitive detection technology [22]. We can extend these scaffolds to other polymerizing molecules so that AHNP fusions with dimeric, trimeric or tetrameric scaffolds may lead to new fully humanized recombinant species suitable for the diagnosis and treatment of p185^{her2/neu}-related tumors.

ASA functions in the same way as the parental antibody which is to promote signaling-defective receptor ensembles [6]. Of note signaling-defective receptor ensembles (*inactive tetrameric complexes*) appear to operationally behave in a manner similar to defective *misaligned dimers* induced by AHNP (Fig. 1C and D). Therefore a unifying principle of disabled receptor kinases caused by antibody or peptide induced ectodomain alterations is one of the bases for the targeted therapeutic process.

Our experiments serve as a defining principle to create new forms of antibody-surrogate molecules in a modular fashion. Biologically active peptides can be linked to a suitable scaffold upon which multiple copies of relevant CDRs are disposed. A small biologically active synthetic peptide can be engineered to enhance functional aspects by structural oligomerization and can be produced recombinantly using bacterial expression.

3.2. Targeting erbB receptor dimerization interface using peptidomimetics

Early studies from our laboratory established that dimer formation was important for p185^{Her2/neu} activation and that mutations in the transmembrane region facilitated dimers and kinase activation [23]. Studies primarily from our laboratory and David Stern's showed that heteromeric kinases could also be induced [24–26]. The EGF-like growth factors bind to the ectodomain of EGFR and other receptors of the same family, leading to the formation of homo- and heterodimers [27,28].

Dimerization consequently stimulates the intrinsic tyrosine kinase activity of the receptors, triggering tyrosine autophosphorylation in the cytoplasmic domain. Phosphorylated tyrosine residues then serve as docking sites for intracellular signaling molecules involved in the regulation of signaling cascades [29–34].

We observed that co-expression of p185^{her2/neu} with EGFR in NIH 3T3 fibroblasts augmented the effects of EGF on the transformed phenotype [25]. Our laboratory also showed that heterodimers were far more active than homodimers of either p185^{c-neu} or EGFR [35]. Numerous studies have confirmed the enhanced transforming abilities of erbB heterodimers [36–42] and found that erbB heteromers are a dominant mediator of malignant properties and also are relevant to the emergence of resistance to agents which target only one member of the erbB family.

Targeting protein-protein interaction (dimerization) surfaces is a promising approach for rational drug design against heteromeric receptors ([43,44], Synthetic peptides and

peptidomimetics that disrupt protein-protein interactions act as inhibitors of HIV-1 protease [45], HIV-1 reverse transcriptase [46], herpes simplex virus ribonucleotide reductase [47], and thymidilate synthase.

We identified distinct loops within the C-terminal part of the subdomain IV as a receptor–receptor interaction site for the erbB receptors. Next we created a peptide mimetic of this loop and demonstrated that this rationally designed loop mimetic was able to target homomeric and heteromeric dimerization interfaces.

Dose-dependent inhibition of erbB receptor dimerization was observed using this loop mimetic in cell lines transfected with different combinations of erbB receptors [48].

Our structural models for dimeric erbB receptors were used to create rationally designed loop mimetic constrained peptides. These species mimicked the potential dimerization site in subdomain IV of p185^{her2/neu}. In surface plasmon resonance studies, our designed peptide mimetics have been shown to selectively bind to the p185^{her2/neu} subdomain IV with submicromolar affinities [48].

4. Trans inhibitory mutants of p185^{her2/neu}

Receptor dimerization is a dominant event for signaling and transformation mediated by the erbB family tyrosine kinases. Because p185^{her2/neu} is the preferred heterodimerization partner of the other erbB family members, we selectively targeted erbB receptors using a kinase deletion mutant consisting of the ectodomain of the p185^{her2/neu} and the transmembrane region but without an endodomain. Species lacking a functional endodomain are still able to lead to the formation of heterodimers while ectodomain lacking forms cannot form dimers. Therefore it is the ectodomain which is most important for dimer formation. We have shown that the endodomain deleted form of p185^{her2/neu}, called T691, can form kinase inactive heterodimers with EGFR or other erbB receptors and thus acts to “trans inhibit” erbB-dependent phenotypes (Fig. 3) [35,49–51].

For example, in EGFR positive U87MG human glioblastoma cells, expression of the p185^{her2/neu} ectodomain inhibits EGF-, but not platelet-derived growth factor-induced DNA synthesis; inhibits cell proliferation in the presence of EGF, but not platelet-derived growth factor; inhibits the ability of U87MG to form colonies in soft agar; and inhibits tumor growth in athymic mice [51].

Our studies established that trans receptor inhibition can abrogate abnormal growth of these tumors. This leads to a general approach for erbB receptor-specific trans inhibition of human neoplasia. These receptor-based inhibitory strategies exploit the thermodynamic preference for erbB ectodomains to heterodimerize, thereby creating erbB receptor assemblies which are defective in signaling and do not internalize. Pharmaceuticals that mimic the p185^{her2/neu} ectodomain may therefore have important therapeutic applications in advanced human malignancies expressing erbB receptors. Our studies also define a rationale for the application of the p185^{her2/neu} ectodomain in gene therapy approaches to human malignant glioma and to other systemic epithelial malignancies expressing erbB family receptors.

5. Small molecules

The intracellular domain of the erbB family receptors includes the tyrosine kinase domain (TKD). TKD is essential for the activation of the receptor and the consequent induction of the downstream pathways that regulate the cell proliferation and other critical functions. The EGFR kinase domain crystal structure was solved by Stamos et al. [52]. The structure shows a bilobate arrangement including an N-lobe, an activation loop and a C-lobe. ATP and substrate bind to the active site of kinase domain and the substrate can be phosphorylated in concomitance with the hydrolysis of ATP. The C-terminal tail of TKD contains several important tyrosine residues whose state of phosphorylation is critical for downstream signal cascades.

Several small molecule tyrosine kinase inhibitors (TKIs) have recently been developed and many of them have already been approved by FDA and used in clinics. Lapatinib (specific to both EGFR and p185^{her2/neu}), Gefitinib (specific to EGFR) and Erlotinib (specific to EGFR) are examples of the most successful reversible kinase inhibitors of the erbB family receptors. The clinical response to these small molecule inhibitors is highly dependent upon certain mutations in the kinase domain [53–55]. Despite significant initial effects observed in patients with mutant EGFR following TKI treatment, most patients eventually develop resistant secondary mutations after a few months of treatment [56]. In addition, for irreversible kinase inhibitors that form a covalent disulfide bond with a cysteine residue (Cys773) in the kinase domain [57], limited success is observed because of the toxicity due to concurrent inhibition of wild type EGFR [58].

Recently we have developed small organic compounds targeting the intracellular kinase domain of EGFR. The compounds can bind to the intracellular domain and mimic the effects produced by the drug sensitizing mutations (Greene MI, Berezov A, unpublished data). This new species represents a novel type of noncompetitive anti-EGFR kinase small molecule inhibitor. We believe that this type of compounds can provide new insights into kinase targeting molecules for cancer and other diseases.

6. Summary

We discovered the activity of anti neu monoclonal antibody targeted therapies in the early 1980s. Now anti-erbB receptor therapy plays an important role in cancer therapy. For decades, we and others have been focused on developing novel therapeutics targeting erbB family tyrosine kinases. Although mAbs and small molecule inhibitors have shown early clinical promises, the mechanisms of action of these therapeutics remain complex and not fully understood. There is still a need to develop more effective receptor-targeting entities and to improve the understanding of their mechanisms of action.

We have tried to define the first order principles to explain ectodomain mediated disabling of erbB receptors. Our laboratory described several operational mechanisms effected by mAbs. These include receptor downregulation, cell cycle arrest, angiogenesis inhibition and facilitation of antibody-dependent cell-mediated cytotoxicity (ADCC).

Endodomain targeted therapy is different and its principles are distinct. mAb alone cause p185^{her2/neu} ubiquitination and degradation of the receptor. However the small molecule TKI lapatinib inhibited p185^{her2/neu} phosphorylation, prevents receptor ubiquitination and degradation, which in turn result in accumulation of stabilized receptor monomer and inactive receptor dimers at cell surface [59]. Kinase activation may be a required step for receptor ubiquitination and degradation, and small molecule TKIs prevent receptor down regulation as a consequence. These findings support combinations of anti-receptor mAb and a receptor-stabilizing TKI in receptor targeted therapy since their modes of action may improve disabling of erbB receptor functions.

Moreover, the treatment regimen that combines erbB targeted therapy with conventional chemotherapy will need to be further explored and optimized. Finding the optimal combinations of receptor targeting biologics and low molecular weight compounds is necessary. In addition identification of predictive biomarkers for drug response, will be useful in monitoring the effectiveness of targeted therapeutics in converting cancer from a lethal process into a chronic disease treatable with less toxic therapies than chemotherapy and high dose irradiation.

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Fig. 1.

Mechanisms of function of therapeutic agents targeting ectodomains of receptors. Receptor ectodomains were represented by circular disks, in which the dimerization interfaces were colored by gray. (A) Monoclonal antibodies 4D5 and 7.16.4 can promote the formation of functionally defective receptor tetramers; (B) monoclonal antibody chA21 appears to cross-link dimers of the receptors to form large defective receptor complexes which are internalized; (C) peptide mimetics AHNP (antibody mimetic) and S22 (dimerization-blocking peptide) can interfere with receptor dimers; (D) like the parental antibody, ASA may also promote the formation of defective receptor tetramers.

Fig. 2.
Three-dimensional structure of AHNP as determined by NMR.

Fig. 3.

Representations of T691, a trans inhibitory mutant of p185^{her2/neu}. Receptor ectodomains were represented by circular disks, in which the dimerization interfaces were colored by gray. Intracellular kinase domains of the receptors were represented by two filled ellipses, and transmembrane domain was represented by lines.