

Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment

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Abstract

The ErbB receptors and their cognate ligands that belong to the epidermal growth factor (EGF) family of peptides are involved in the pathogenesis of different types of carcinomas. In fact, the ErbB receptors and the EGF-like growth factors are frequently expressed in human tumors. These proteins form a complex system that regulates the proliferation and the survival of cancer cells. Therefore, ErbB receptors and their ligands might represent suitable targets for novel therapeutic approaches in human carcinomas. In this regard, different target-based agents that are directed against the ErbB receptors have been developed in the past two decades. One of these compounds, the humanized anti-ErbB-2 monoclonal antibody trastuzumab has been approved for the treatment of patients with metastatic breast cancer. The anti-EGF receptor (EGFR) antibody C225, as well as EGFR tyrosine kinase inhibitors ZD1839 and OSI-774 are currently in phase III clinical development. Several other ErbB tyrosine kinase inhibitors are in phase I/II studies. These compounds have generally been shown to have an acceptable toxicity profile and promising anti-tumor activity in heavily pretreated patients. The mechanisms of action of these compounds, as well as the potential therapeutic strategies to improve their efficacy are discussed in this review with particular regard to the combinations of anti-ErbB agents with cytotoxic drugs, or combinations of different ErbB-targeting agents.

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Introduction

Identification of the mechanisms that are involved in regulating proliferation and survival of tumor cells is leading to the development of novel therapeutic approaches to human carcinomas. In fact, several different agents that are directed against specific intracellular or extracellular signaling molecules expressed in tumor cells are currently in clinical development. In most of the cases, the targets of these agents are also expressed in normal cells. However, the toxicity profile of these molecules is generally acceptable, and the side-effects are much more tolerable as compared with conventional cytotoxic agents. Therefore, the use of these types of drugs will allow the treatment of patients who could not tolerate therapies with conventional anti-tumor drugs. Furthermore, several reports have shown that these agents might be effective in patients who do not respond to conventional therapy.

Growth factors and receptors are ideal targets for this novel therapeutic approach. In fact, one important feature of transformed cells is that they exhibit a reduced requirement for exogenously supplied growth factors to maintain a high rate of proliferation as compared with their normal counterparts. This relaxation in growth factor dependency may be due in part to the ability of tumor cells to produce high levels of peptide growth factors and/or growth factor receptors. The tumor-derived growth factors may function through intracrine, paracrine, juxtacrine and/or autocrine pathways to control cell proliferation and survival (Sporn & Roberts 1992). Growth factors are also involved in regulating angiogenesis and formation of metastasis through interaction with the surrounding stromal cells. In this regard, expression of a high number of receptors on the surface of tumor cells can also increase their sensitivity to host-derived growth factors.

Tumor cells are able to synthesize and to respond to a number of different peptide growth factors (Aaronson 1991).

Table 1 The ErbB receptors and their cognate ligands

Receptor	Ligands
EGFR	Epidermal growth factor (EGF) Transforming growth factor- α (TGF α) Amphiregulin (AR) Epiregulin (EP) Betacellulin (BTC) Heparin-binding EGF-like growth factor (HB-EGF)
ErbB-2	Unknown
ErbB-3	Neuregulin (NRG) 1/hereregulin (HRG) isoforms NRG-2 α and β
ErbB-4	NRG-1/HRG isoforms NRG-2 α and β NRG-3 NRG-4 Tomoregulin HB-EGF BTC EP
Unknown	Cripto-1 (CR-1) Criptin

Among these, it has long been established that the epidermal growth factor (EGF)-related peptides and their cognate receptors might contribute to the growth of tumor cells (Normanno *et al.* 2001). In fact, the EGF superfamily includes several proteins of diverse function that are structurally and functionally related (Normanno *et al.* 2001). Some of these proteins can indeed function as peptide growth factors by binding to specific transmembrane tyrosine kinase receptors (Table 1). In particular, the EGF-like proteins bind and activate receptors of the ErbB family, with the only exception being the peptides of the EGF-Cripto-FRL-1-Cryptic (CFC) subfamily, which function in part through a type I ALK-4 activin receptor.

In this review article, we shall briefly summarize the complex interactions existing between the growth factors of the EGF family, the ErbB receptors and the intracellular signal transduction pathways that are activated by ligand-receptor interactions. We shall discuss the pre-clinical findings on the mechanism of action of these agents, as well as the mechanisms that are potentially involved in the anti-tumor effects of combinations of anti-ErbB agents and chemotherapeutic agents. Finally, we shall describe the therapeutic strategies in which these novel drugs might be employed.

The ErbB receptor/ligand network

The structure and function of the ErbB receptors and of their cognate ligands have been described in detail elsewhere (Mason & Gullick 1995, Schlessinger 2000, Normanno *et al.*

2001, Prenzel *et al.* 2001). However, we briefly summarize the information that is necessary to understand the rationale of anti-ErbB therapies, and the mechanism of action of anti-ErbB drugs.

The ErbB receptor family includes four members, the EGF receptor (EGFR) (also known as ErbB-1/HER1), ErbB-2/Neu/HER2, ErbB-3/HER3 and ErbB-4/HER4, which belong to the type I receptor tyrosine kinase family (Mason & Gullick 1995). In fact, these receptors are single amino acid chain proteins which span the cell membrane once. Each of these proteins possesses three different domains: the extracellular domain which is involved in recognizing and binding the ligands that are able to activate the receptor, the transmembrane-spanning sequence which is involved in interaction between receptors, and the intracellular domain in which resides the enzymatic activity of the tyrosine kinase that is able to phosphorylate tyrosine residues on different intracellular adaptor proteins (Mason & Gullick 1995). The cytoplasmic domain also consists of a carboxy-terminal tail containing tyrosine autophosphorylation sites which link these receptors to proteins containing Src homology 2 and phosphotyrosine-binding domain motifs (Mason & Gullick 1995, Schlessinger 2000, Van Zoelen *et al.* 2000). The members of the ErbB family have a similar structure with a high degree of homology in the tyrosine kinase domain (Mason & Gullick 1995, Van Zoelen *et al.* 2000). The high homology between these proteins makes possible the synthesis of inhibitors that are able to bind two or more receptors. In contrast, the extracellular domains are less conserved among the four receptors, which is indicative of different specificity in ligand binding.

The key event for the activation of the ErbB receptors is the binding of a peptide growth factor to the extracellular domain of the receptor. The growth factors that bind and activate the ErbB receptors belong to the EGF family of peptide growth factors (Table 1) (Normanno *et al.* 2001). This family includes EGF, TGF α , AR, HB-EGF, BTC, EP and tomoregulin. In addition, this family contains the NRG subfamily which consists of four different genes: NRG-1, NRG-2, NRG-3 and NRG-4. The NRG-1 family includes the rat neu differentiation factor, the HRGs, glial growth factors, acetylcholine receptor-inducing activity and sensory motor neuron-derived growth factor that have been identified in different biological systems and have been given alternative names (Peles & Yarden 1993, Pinkas-Kramarski *et al.* 1994). More recently, an EGF-CFC subfamily which includes the human CR-1 and criptin has been also described (Salomon *et al.* 2000). Most of these growth factors are synthesized as cell membrane-associated precursors that are biologically active and can interact with receptors on adjacent cells through a juxtacrine pathway (Massagué & Pandiella 1993). In this regard, some of these cell-associated precursors can function as cell-cell adhesion molecules that regulate migration and colonization of specific organs during metastasis (Campbell & Bork 1993, Massagué & Pandiella 1993).

We can distinguish within the EGF-like family of growth factors three classes of ligands that can bind and activate distinct sets of individual receptors (Olayioye *et al.* 2000) (Table 1). The first group consists of EGF, AR and TGF α which bind exclusively to the EGFR (Shoyab *et al.* 1989, Carpenter & Cohen 1990, Derynck 1992). HB-EGF and BTC can efficiently interact with the EGFR and ErbB-4 (Riese *et al.* 1996, Elenius *et al.* 1997). EP appears to be a broad spectrum receptor ligand. In fact, EP stimulates homodimers of both EGFR and ErbB-4, and it also activates all possible heterodimeric ErbB complexes (Shelly *et al.* 1998). Finally the NRGs/HRGs can bind to either ErbB-3 or ErbB-4. In particular, NRG-1 and NRG-2 bind both ErbB-3 and ErbB-4, whereas NRG-3 and NRG-4 bind ErbB-4 but not ErbB-3 (Pinkas-Kramarski *et al.* 1996, Zhang *et al.* 1997, Crovello *et al.* 1998, Harari *et al.* 1999).

There are two exceptions within the ErbB family. ErbB-2 has a powerful tyrosine kinase activity, but it is not able to bind any known ligand (Normanno *et al.* 2001). Reciprocally, ErbB-3 binds ligands of the HRG subfamily of peptides, and it has an impaired tyrosine kinase domain (Guy *et al.* 1994). However, both ErbB-2 and ErbB-3 can take part in signal transduction through formation of heterodimers with the other ErbB receptors. In fact, receptor dimerization, which occurs following binding of a ligand to its specific receptor, is essential for the activation of the tyrosine kinase activity of the receptors and for the subsequent generation of an intracellular signal. Individual receptor pairings can consist of two molecules of the same type, called homodimers, or two molecules of different types, which has been termed a heterodimer. All possible homo- and heterodimeric receptor complexes between members of the ErbB family have been identified in different systems (Olayioye *et al.* 2000, Schlessinger 2000, Gullick 2001). Through this mechanism, ErbB-2 and ErbB-3 can complement each other's deficiencies: a heterodimeric complex containing an orphan receptor (ErbB-2) and an inactive tyrosine kinase receptor (ErbB-3) can form the most potent ErbB signaling complex in terms of cell proliferation and transformation (Alimandi *et al.* 1995). In this context, ErbB-2 seems to be the preferred dimerization partner for all the other ErbB receptors (Tzahar *et al.* 1996, Graus-Porta *et al.* 1997). The three heterodimers that are formed most frequently are ErbB-2/ErbB-3, ErbB-2/ErbB-4 and ErbB1/ErbB-4. However, the different ability to form homo- and heterodimers is also dictated by the levels of expression of these receptors. It has been also suggested that spontaneous dimerization of ErbB-2 might occur in cells that express high levels of this receptor (Samanta *et al.* 1994, Muthuswamy *et al.* 1999). However, these data have been obtained by using synthetic ligands. In fact, this phenomenon has not been formally proven to occur in human cancer.

The four ErbB receptors share most of the intracellular second messengers generated when they are activated (Olayioye *et al.* 2000). These include adaptor proteins such

as Shc and Grb2, which couple the ErbB receptors to the *ras/raff*/mitogen-activated protein kinase (MAPK) pathway, kinases such as *c-src* and phosphatidylinositol 3-kinase (PI3K), and protein tyrosine phosphatases such as SHP1 and SHP2 (Olayioye *et al.* 2000). However, there are also examples of preferential activation of specific pathways. For example, PI3K is activated in particular by ErbB-3 due to the presence in its intracellular domain of multiple binding sites for the p85 PI3K regulatory subunit (Prigent & Gullick 1994). ErbB-4 can be expressed as two variants which either possess or lack a 16 amino acid sequence in the intracellular domain that functions as a binding site for PI3K (Elenius *et al.* 1999). Finally, formation of heterodimers might significantly affect the duration and the 'quality' of the ErbB signaling. In fact, the rate of internalization of the receptors regulates the length of the stimulus that is carried by the activated receptors. This process depends on the ligands that bind to a specific receptor. In this regard, it has been shown that EGF-driven homodimers of the EGFR are rapidly degraded, whereas TGF α -induced homodimers are recycled to the cell surface, thereby resulting in enhanced signaling (Lenferink *et al.* 1998). However, it has also been shown that receptor heterodimerization can affect this phenomenon. In fact, all the ErbB receptors other than the EGFR are endocytosis impaired (Baulida *et al.* 1996). Therefore, heterodimers which contain ErbB-2, ErbB-3 or ErbB-4 might have reduced receptor internalization and degradation as compared with EGFR homodimers. In this context, it has been demonstrated that EGF-driven heterodimers of the EGFR with either ErbB-2 or ErbB-3 dissociate in the early endosome (Lenferink *et al.* 1998). In the presence of either co-receptor, EGFR is recycled to the cell surface and its signaling is enhanced as compared with EGFR homodimers. Furthermore, NRG-1-activated EGFR/ErbB-4 heterodimers have delayed internalization characteristics as compared with the rapid internalization of EGF-activated EGFR homodimers (Olayioye *et al.* 1998). Finally, it has been demonstrated that ErbB receptors can acquire different signaling properties following dimerization with different partners (Olayioye *et al.* 1998, 1999).

The ErbB receptors and their ligands are frequently expressed in human carcinomas and therefore might play an important role in the pathogenesis of these diseases. In particular, expression of the EGFR and of its ligands has been demonstrated to occur with high frequency in a majority of human carcinomas (Table 2) (for review see Salomon *et al.* 1995, Prenzel *et al.* 2001). Expression of ErbB-2 in human carcinomas has also been extensively investigated in the last two decades (for review see Salomon *et al.* 1995, Prenzel *et al.* 2001) (Table 2). Expression of this receptor occurs at relatively lower frequency as compared with the EGFR. In fact, it has been demonstrated that ErbB-2 protein expression and/or gene amplification occurs in 20–40% of several carcinoma types. More recently, expression of both ErbB-3 and

Table 2 Expression of ErbB receptors in human carcinomas

Type of cancer	EGFR (%)	ErbB-2 (%)	ErbB-3 (%)	ErbB-4 (%)
Lung	40–80	18–60	25–85	NA*
Breast	14–91	9–39	22–90	82
Stomach	33–74	8–40	35–100	NA
Colon	25–77	11–20	65–89	NA
Esophagus	43–89	7–64	64	NA
Liver	47–68	0–29	84	61
Pancreas	30–50	19–45	57–63	81
Prostate	40–80	40–80	22–96	NA
Kidney	50–90	0–40	0	NA
Bladder	35–86	9–50	30–56	30
Ovary	35–70	8–32	85	93
Head and neck	36–100	17–53	81	28–69

*NA, not assessed.

ErbB-4 in human primary carcinomas has been demonstrated. In particular, several reports have described the expression of ErbB-3 in human carcinomas by using immunohistochemical techniques (Lemoine *et al.* 1992, Poller *et al.* 1992, Sanidas *et al.* 1993, Gasparini *et al.* 1994, Myers *et al.* 1994, Friess *et al.* 1995, 1999, Simpson *et al.* 1995b, Rajkumar *et al.* 1996, Travis *et al.* 1996, Fontanini *et al.* 1998, Maurer *et al.* 1998, Naidu *et al.* 1998, Slesak *et al.* 1998, Xia *et al.* 1999, Kawesha *et al.* 2000, Porebska *et al.* 2000, Chow *et al.* 2001, Ito *et al.* 2001) (Table 2). The levels and frequency of expression of ErbB-3 in human carcinomas are generally comparable with the EGFR. Immunoreactive ErbB-4 or specific transcripts have been found in different tumors, such as breast, ovarian, squamous cell, esophageal, bladder and pancreatic cancer (Graber *et al.* 1999, Xia *et al.* 1999, Srinivasan *et al.* 2000, Bei *et al.* 2001, Chow *et al.* 2001, Gilmour *et al.* 2001, Ito *et al.* 2001, Suo *et al.* 2002) (Table 2). These data clearly suggest that co-expression of two or more ErbB receptors frequently occurs in human carcinomas. Likewise, it has been previously demonstrated that co-expression of different EGF-like peptides is a common phenomenon in human carcinogenesis (for review see Normanno *et al.* 2001). For example, co-expression of TGF α , AR and/or CR-1 occurs in a majority of human colon, breast, lung, ovarian and gastric carcinomas (Saeki *et al.* 1992, 1994, Qi *et al.* 1994, Normanno *et al.* 1995, Panico *et al.* 1996, Fontanini *et al.* 1998, D'Antonio *et al.* 2002). These observations are important for therapeutic approaches, as we shall discuss later.

The anti-ErbB target-based agents: pre-clinical development and clinical studies with single agents

The above-mentioned findings clearly suggest that the ErbB receptors and their cognate ligands represent suitable targets

for experimental therapeutic approaches in human tumors. In this context, there are several sites in growth factor-regulated pathways where therapeutic intervention might be possible: the synthesis and the secretion of the growth factor, binding to the receptor, and the synthesis and/or the activation of the receptor and proteins involved in the intracellular signal transduction. Several different agents directed against receptors and ligands have been synthesized in the past two decades: anti-receptor- or anti-growth-factor blocking monoclonal antibodies, bacterial or fungal toxins conjugated to ligands or antibodies, and antisense oligonucleotides and tyrosine or serine kinase inhibitors (Fig. 1). However, this discussion will focus mainly on drugs that have already been approved for patient treatment or that are in an advanced phase of clinical study, namely monoclonal antibodies and tyrosine kinase inhibitors (Table 3).

Monoclonal antibodies

The first anti-ErbB receptor drug that has been approved for treatment of cancer patients is the anti-ErbB-2 humanized monoclonal antibody trastuzumab (Herceptin). As a matter of fact, this drug represents the first example of a new era of anti-cancer therapy based on the use of target-selective drugs. Trastuzumab was developed at Genentech (San Francisco, CA, USA) starting from the murine anti-ErbB-2 antibody 4D5 (Carter *et al.* 1992). In this regard, trastuzumab has a higher binding affinity for ErbB-2 as compared with the murine 4D5, is able to inhibit the *in vitro* and *in vivo* growth of tumor cells overexpressing ErbB-2, and is much more efficient in supporting antibody-dependent cellular cytotoxicity against human tumor cell lines in the presence of human peripheral mononuclear cells (Carter *et al.* 1992, Baly *et al.* 1997, Pegram *et al.* 1997a). The mechanism by which anti-ErbB-2 antibodies inhibit tumor cell proliferation is not well defined (Sliwkowski *et al.* 1999). The 4D5 antibody has been reported to either stimulate or reduce the tyrosine phosphorylation of ErbB-2 (Kumar *et al.* 1991, Scott *et al.* 1991, Sliwkowski *et al.* 1999, Lane *et al.* 2000). However, both 4D5 and trastuzumab induce a marked downregulation of ErbB-2 (Hudziak *et al.* 1989, Kumar *et al.* 1991, Sarup *et al.* 1991). Trastuzumab also has a partial ability to disrupt the formation of ErbB-2/ErbB-3 and ErbB-2/ErbB-4 heterodimers, suggesting that it might be able to impair signaling through other ErbB receptors (Klapper *et al.* 1997). It has been shown that trastuzumab and 4D5 induced G1 arrest of cell cycle progression in breast cancer cells (Sliwkowski *et al.* 1999). In particular, Lane *et al.* (2000) showed that treatment with 4D5 resulted in increased levels of p27 in BT-474 cells but not in Sk-Br-3 human breast cancer cells. By using antisense oligonucleotides, they have also demonstrated that an increase in p27 is not necessary for G1 blockade induced by 4D5 (Lane *et al.* 2000). They hypothesized that a reduction in the levels of sequestration proteins (cyclin D1)

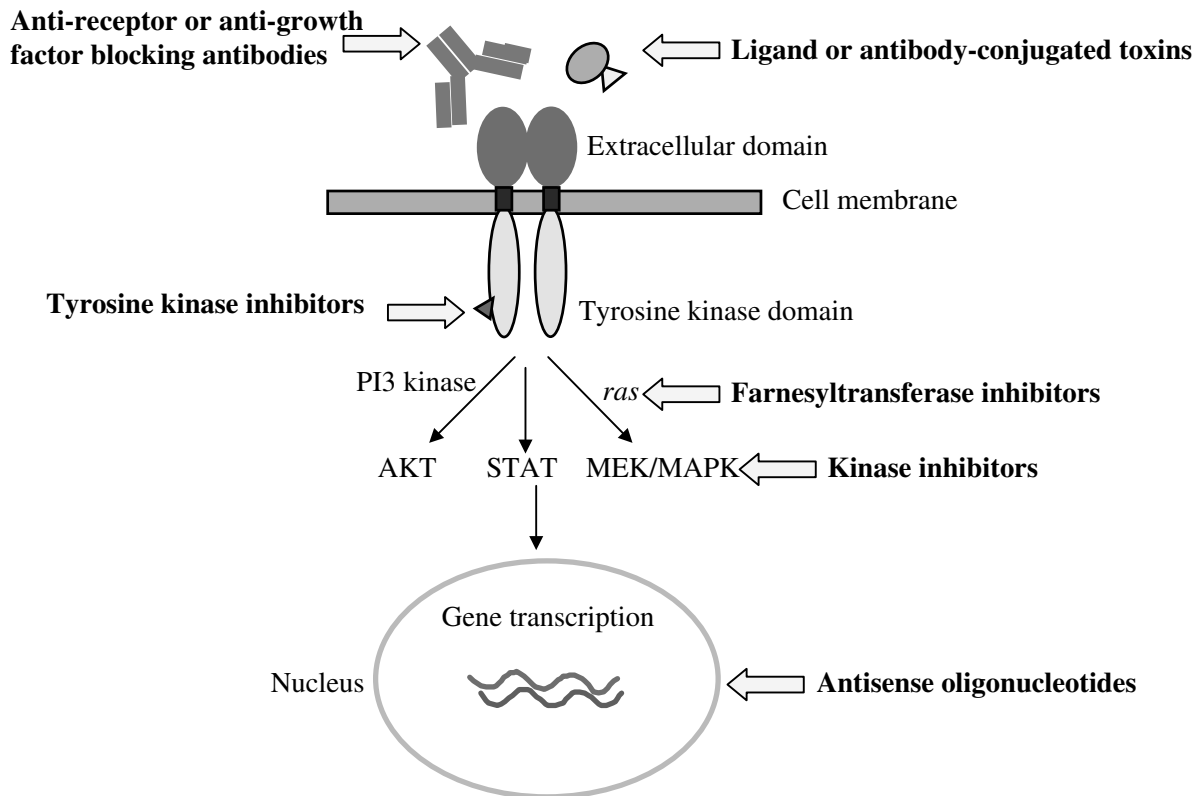


Figure 1 Potential sites for therapeutic intervention in growth factor-regulated pathways. The therapeutic agents that have been employed to interfere with the different steps of growth factor-induced signaling are listed.

Table 3 A summary of the anti-ErbB drugs that are in most advanced phases of clinical development

Drug	Target	Description	Trial status
Trastuzumab	ErbB-2	Humanized antibody	Approved
IMC-C225	EGFR	Humanized antibody	Phase III
ABX-EGF	EGFR	Humanized antibody	Phase II
ZD1839	EGFR	Quinazoline reversible TKI	Phase III
OSI-774	EGFR	Quinazoline reversible TKI	Phase III
CL-1033	ErbB receptors	Quinazoline irreversible TKI	Phase I
PKI-166	EGFR/ErbB-2	Pyrrolo-pyrimidine reversible TKI	Phase I
EB-569	EGFR	Quinazoline irreversible TKI	Phase I
GW-2016	EGFR/ErbB-2	Quinazoline reversible TKI	Phase I

TKI, tyrosine kinase inhibitor.

leads to the redirection of p27 on cyclin dependent kinase Cdk2 complexes and to a G1 block. However, these data have not been confirmed by independent research groups. In this regard, Sliwkowski *et al.* (1999) showed that an increase in p27 levels occurs following treatment of Sk-Br-3 breast cancer cells with trastuzumab. Furthermore, it has been demonstrated that treatment with trastuzumab resulted in translocation of p27 from cytosol to cell nuclei in BT-474 cells (Yakes *et al.* 2002). However, this treatment also induced an increase in p27 protein levels, and antisense oligonucleotides directed against p27 significantly reduced the ability of tras-

tuzumab to reduce the proportion of cells in S-phase (Yakes *et al.* 2002). Taken together, these data clearly suggest that both the increase of p27 and the re-localization of p27 might be involved in the trastuzumab-induced G1 growth arrest. More recent data have shed light on the mechanism through which trastuzumab might block cell cycle progression. In fact, both the PI3K/Akt and MAPK pathways have been hypothesized to be involved in modulating p27 expression and/or function in ErbB-2 overexpressing cells (Lenferink *et al.* 2001). However, it has been recently demonstrated by two independent groups that Akt is able to directly phosphorylate

p27 (Shin *et al.* 2002, Viglietto *et al.* 2002). This phenomenon leads to cytoplasmic retention of p27 which precludes p27-induced G1 arrest. Therefore, the ability of trastuzumab to reduce Akt activation might represent a key event for its anti-tumor, cytostatic effect. Finally, trastuzumab has been shown to be a potent inhibitor of ErbB-2 cleavage on the cell surface membrane, and to prevent the generation of membrane-bound phosphorylated truncated receptor (p95) (Molina *et al.* 2001).

Phase II clinical trials have demonstrated the efficacy of trastuzumab in patients with metastatic breast cancer overexpressing ErbB-2. Two studies involved patients who failed prior chemotherapy. The first study enrolled 46 patients with an overall response rate of 11.6% (Baselga *et al.* 1996). In a larger study involving 222 patients, the results showed an objective response rate of 15% (Cobleigh *et al.* 1999). In a more recent study, an overall response rate of 26% was observed within a population of 114 ErbB-2-positive, untreated breast cancer patients (Vogel *et al.* 2002). Interestingly, the response rate in patients with the higher level of ErbB-2 expression (3+) was 35% versus none in ErbB-2 2+ tumors. Analogously, the response rate in patients with and without ErbB-2 amplification as assessed by fluorescent *in situ* hybridization (FISH) was 34% and 7% respectively. These data clearly demonstrate that trastuzumab is active in patients with high levels of expression of ErbB-2 (3+ by immunohistochemistry or FISH positive).

A series of anti-EGFR monoclonal antibodies with inhibitory activity against cells expressing the target receptor has been developed. Several mechanisms are probably involved in the anti-tumor activity of these compounds. We shall focus this discussion on the anti-EGFR 225 antibody that has reached clinical development. The 225 antibody is able to induce dimerization, internalization and downregulation of the EGFR (Fan *et al.* 1994). More importantly, this antibody is able to block the activation of the tyrosine kinase domain of the EGFR following stimulation with a specific ligand (Kawamoto *et al.* 1983, Gill *et al.* 1984, Goldstein *et al.* 1995). As a consequence of receptor blockade, treatment with the 225 antibody produces an increase in the levels of p27kip bound to CDK, and therefore a slowing of the proliferation and arrest in the G1 phase of the cell cycle (Wu *et al.* 1996, Fan *et al.* 1997). This blockade is not usually followed by apoptosis, although induction of programmed cell death was observed in DiFi colon carcinoma cells (Wu *et al.* 1995). In addition, blockade of the EGFR with monoclonal antibodies results in a significant inhibition of neo-angiogenesis. This phenomenon seems to be related to the reduction in the synthesis of angiogenic factors such as interleukin-8 (IL-8), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in tumor cells following treatment with anti-EGFR monoclonal antibodies (Ciardiello *et al.* 1996, Petit *et al.* 1997, Perrotte *et al.* 1999, Bruns *et al.* 2000a). Based on the promising anti-tumor

activity, the 225 antibody has been selected for clinical development. In order to obviate the immune response against the murine antibodies, a chimeric human: murine version of the 225 antibody, IMC-C225 (C225), was generated (Goldstein *et al.* 1995). Three phase I studies with this drug alone or in combination with cytotoxic drugs have been conducted in order to define the optimal dose (Baselga *et al.* 2000b). In particular, results from these trials have defined an initial dose of 400 or 500 mg/m², followed by 250 mg/m² weekly thereafter, as optimum for continuous saturation of C225 clearance and blockade of the EGFR. At this dose level, a significant reduction in levels of activation of MAPK and in keratinocyte Ki67 proliferation indexes was observed in the skin of patients treated with C225 (Albanell *et al.* 2001). Clinical activity of C225 in phase I clinical studies in which patients were treated with C225 alone has been observed (Baselga *et al.* 2000b). In particular, treatment with single agent IMC-C225 in two different phase I clinical trials, in which IMC-C225 was administered as a single i.v. infusion or for 4 weeks, resulted in disease stabilization at 4 weeks for seven of twelve (58%) and eleven of sixteen (69%) cancer patients respectively. Most of the phase II studies with C225 thereafter have been conducted in combination with chemotherapy or radiotherapy.

A fully humanized anti-EGFR monoclonal antibody, ABX-EGF, is currently in phase II clinical development. Preliminary data have shown that this antibody is active in renal carcinoma patients (Schwartz *et al.* 2002).

Tyrosine kinase inhibitors

The tyrosine kinase activity of the ErbB receptors is required for the biological responses induced by ligand-receptor interactions. Several tyrosine kinase inhibitors have been synthesized in the last decade. These compounds directly inhibit tyrosine kinase phosphorylation by physical interaction with either the ATP and/or the enzyme substrate-binding sites (Klohs *et al.* 1997, Fry 2000, Morin 2000). Some of these molecules, such as tyrphostins, are able to inhibit the tyrosine kinase activity of different types of receptors. More recently, compounds that specifically inhibit the EGFR tyrosine kinase have been generated (Klohs *et al.* 1997). Inhibitors of the EGFR are currently dominated by three series of compounds, which include quinazolines, pyridopyrimidines and pyrrolopyrimidines. Some of these molecules are currently in clinical development (Table 3).

ZD1839 (Iressa) is an orally active, specific EGFR tyrosine kinase inhibitor. ZD1839 is a reversible inhibitor of the EGFR that functions by competing with ATP in binding to the tyrosine kinase domain of the receptor. It can inhibit the tyrosine kinase activity of EGFR extracted from human A431 vulval squamous carcinoma cells with an IC₅₀ of 0.023-0.079 mM (Barker *et al.* 2001). ZD1839 is able to block the *in vitro* and/or *in vivo* growth of a wide range of

EGFR-expressing human cancer cell lines (Ciardiello *et al.* 2000, 2001, Anderson *et al.* 2001, Moasser *et al.* 2001, Moulder *et al.* 2001, Magne *et al.* 2002b, Normanno *et al.* 2002, Swell *et al.* 2002). The activity of this drug is generally cytostatic, although it was shown to induce apoptosis in several cancer cell lines (Ciardiello *et al.* 2000, Moulder *et al.* 2001, Normanno *et al.* 2002). Recent pre-clinical data have shed light on the mechanism of action of ZD1839 (Moasser *et al.* 2001, Moulder *et al.* 2001, Anido *et al.* 2002, Campiglio *et al.* 2002, Normanno *et al.* 2002). In fact, ZD1839 basically functions by blocking the tyrosine kinase activity of the EGFR. However, it has been shown that its activity does not depend on the levels of expression of the EGFR. In this regard, cell lines with either low or high levels of EGFR expression are sensitive to ZD1839. It has also been shown that ErbB-2-overexpressing cells are highly sensitive to ZD1839. In these cells, ZD1839 treatment significantly reduces the phosphorylation of the EGFR, ErbB-2 and ErbB-3 (Moulder *et al.* 2001, Campiglio *et al.* 2002, Normanno *et al.* 2002). The effects of ZD1839 on the activation of ErbB-2 and ErbB-3 are not direct, but are mediated by the EGFR. In fact, ZD1839 was not able to block the activation of ErbB-2 in cell lines that overexpress ErbB-2 but do not express the EGFR (Moulder *et al.* 2001, Campiglio *et al.* 2002, Normanno *et al.* 2002). Furthermore, since ErbB-3 has no tyrosine kinase activity, it is evident that the reduction of ErbB-3 phosphorylation induced by treatment with ZD1839 is due to a reduced transactivation by either the EGFR or ErbB-2. In this context, it has been demonstrated that ZD1839 induces in target cells the formation of EGFR/ErbB-2 and EGFR/ErbB-3 heterodimers in which the receptors are in an inactive state (Arteaga *et al.* 1997, Anido *et al.* 2002). This phenomenon leads to a reduction of ErbB-2/ErbB-3 heterodimers (Moulder *et al.* 2001, Anido *et al.* 2002). Therefore, ZD1839, a specific EGFR tyrosine kinase inhibitor, is able to affect signal transduction through different ErbB receptors by inducing the EGFR to capture both ErbB-2 and ErbB-3 in inactive dimers. ZD1839 has no effect on the formation of ErbB-2 homodimers which can spontaneously activate. However, since the growth and the survival of ErbB-2-overexpressing cells was significantly affected by treatment with ZD1839 (up to 60% growth inhibition in BT-474 cells), it is evident that transactivation of ErbB-2 in dimers with either the EGFR or ErbB-3 is an important phenomenon in the autonomous growth of these cancer cells (Normanno *et al.* 2002). Finally, it has been shown that the *in vivo* anti-tumor effects of ZD1839 are, at least in part, due to its anti-angiogenic activity. In fact, ZD1839 is able to reduce the synthesis of angiogenic factors (VEGF, IL-8 and bFGF) in tumor cells, and to block the migration and formation of tube-like structures by human microvascular endothelial cells (Ciardiello *et al.* 2001, Hirata *et al.* 2002).

The tolerability of ZD1839 has been evaluated in four phase I dose-escalation studies. A total of 254 patients with

a range of solid malignancies, including 100 patients with non-small cell lung cancer (NSCLC), have been enrolled in these studies (Baselga *et al.* 2000a, Ferry *et al.* 2000, Goss *et al.* 2000, Nakagawa *et al.* 2000). ZD1839 was administered once daily with an intermittent or continuous schedule. Dose-limiting toxicity was predominantly diarrhea with the maximum tolerated dose being reached at 700–800 mg/day, depending on the dose schedule used. The most frequent adverse events were skin changes (mainly acne-like rash) and diarrhea. Encouraging anti-tumor activity was evident in all four trials. In particular, 10/100 patients with NSCLC demonstrated a partial response across a range of doses. The pharmacodynamic effects of ZD1839 were investigated using skin as an EGFR-dependent tissue in patients participating in phase I clinical trials. ZD1839 was shown to inhibit EGFR activation and downstream receptor-dependent processes at doses well below the one producing unacceptable toxicity (Albanell *et al.* 2002). The results from phase I studies of ZD1839 provided a rationale for phase II/III development in NSCLC. Two large randomized, phase II trials, Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL 1) and IDEAL 2, have assessed the tumor response rates, disease-related symptom improvement rates, and safety profiles of two doses of ZD1839 in pretreated patients with NSCLC. Each trial included approximately 200 patients. IDEAL 1 enrolled patients with NSCLC who had failed one or two chemotherapy regimens (at least one platinum-based therapy) (Fukuoka *et al.* 2002). In this study, ZD1839 at 250 mg/day and 500 mg/day demonstrated no difference in the efficacy endpoints measured. The tumor response rates were 18.4% (250 mg/day) and 19.0% (500 mg/day), and the disease control rates were 54.4% (250 mg/day) and 51.4% (500 mg/day). IDEAL 2 investigated the activity and safety of daily oral ZD1839 (250 vs 500 mg/day) in patients with locally advanced or metastatic NSCLC, who failed two or more prior chemotherapy regimens containing platinum and docetaxel (Kris *et al.* 2002). Tumor response rates for the 250 and 500 mg/day ZD1839 groups were 11.8% and 8.8% respectively, whereas 31% and 27% of patients had stable disease in the 250 and 500 mg/day ZD1839 arms. The 1-year survival was 86% in patients with partial response, 44% in patients with stable disease and 8% in patients who experienced progression of the disease during the treatment. Therefore, the activity of ZD1839 as a single agent in third-line therapy of NSCLC patients was similar or even higher as compared with cytotoxic agents such as taxanes. The anti-tumor activity of ZD1839 has also been confirmed in heavily pretreated NSCLC patients who have been treated on a compassionate protocol (Janne *et al.* 2002, Liem *et al.* 2002, Ruckdeschel *et al.* 2002, Soto Parra *et al.* 2002). Although the response rate in these patients was lower than 10%, up to 50% of patients experienced some clinical benefit. Taken together, these results also demonstrated that the frequency of response to treatment with ZD1839 as the single agent was

significantly higher as compared with anti-EGFR monoclonal antibodies, at least in NSCLC patients. The complex mechanism of action of ZD1839, which is able to block the activation of different ErbB receptors, might explain such differences. Finally, clinical responses to treatment with single agent ZD1839 have been recently reported to occur in head and neck cancer, but not in renal carcinoma patients (Cohen *et al.* 2002, Drucker *et al.* 2002).

OSI-774 (Tarceva) is a quinazoline derivative that is able to specifically block the kinase activity of purified EGFR and EGFR autophosphorylation in intact cells with IC₅₀ values of 2 and 210 nmol/l respectively (Moyer *et al.* 1997). Nanomolar concentrations of OSI-774 were demonstrated to inhibit the growth of various cancer cell lines that express the EGFR (Moyer *et al.* 1997). It was also able to induce G1 arrest, accumulation of p27kip1 and apoptosis in DiFi human colon carcinoma cells (Moyer *et al.* 1997). In addition, oral treatment of mice bearing human HN5 head and neck carcinoma xenografts with OSI-774 resulted in a significant inhibition of tumor growth (Pollack *et al.* 1999). This effect was accompanied by a significant reduction of intratumoral EGFR phosphorylation. Two phase I dose-escalation pharmacokinetic trials have been conducted with OSI-774 in patients with advanced solid tumors. One of these studies has been recently published (Hidalgo *et al.* 2001). Like ZD1839, dose-limiting toxicity was diarrhea and/or cutaneous toxicity at doses exceeding 150 mg/day in the continuous, once daily schedule. Therefore, the recommended dose for OSI-774 is 150 mg/day in an uninterrupted schedule. Anti-tumor activity was shown in phase I trials in patients with colorectal, non-small cell lung, prostate, cervical and head and neck carcinomas. These findings have been confirmed by preliminary results of phase II studies. In particular, clinical activity of OSI-774 in a group of 124 pretreated, advanced head and neck cancer patients has been shown (Senzer *et al.* 2001). The overall response rate was 5.6% (7/124), with 39 patients (33.9%) experiencing stable disease. Clinical responses have also been described in 2/34 ovarian carcinoma patients and in 7/57 NSCLC patients in two different phase II trials (Finkler *et al.* 2001, Perez-Soler *et al.* 2001). In both cases, patients had advanced, heavily pretreated disease.

CI-1033 is an orally active 4-anilinoquinazoline that acts as a pan-erbB tyrosine kinase inhibitor (Slichenmyer *et al.* 2001). Drug-related dose-limiting toxicities of grade 3 stomatitis and rash (acneiform and maculopapular) were observed at a dose of 220 mg in a phase I clinical trial (Rinehart *et al.* 2002). However, the maximum tolerated dose was dependent on the schedule of administration. The effects of this compound on Ki67 and p27 expression, as well as on the phosphorylation state of EGFR and ErbB-2 have been described (Zinner *et al.* 2002). A similar toxicity profile has been reported for EKB-569 which is a selective, irreversible inhibitor of the EGFR tyrosine kinase (Hidalgo *et al.* 2002). GW2016 is an additional quinazoline derivative that is able

to block the activation of both the EGFR and ErbB-2, and which is currently in phase I clinical trial (Rusnak *et al.* 2001). Finally, PKI-166 is a pyrrolo-pyrimidine that was initially developed as a reversible inhibitor of the EGFR (Bruns *et al.* 2000b). More recently, it has been shown that this compound is also able to block the enzymatic activity of ErbB-2. The IC₅₀ *in vitro* of PKI-166 for ErbB-2 is 11 times higher as compared with the EGFR (Traxler *et al.* 2001). PKI-166 was indeed able to block the growth of mouse cells engineered to express high levels of ErbB-2 (Brandt *et al.* 2001, Traxler *et al.* 2001). However, it is not clear whether the ability to block ErbB-2 is important for its anti-tumor effect. In fact, the IC₅₀ values of this compound for SK-Br-3 and BT-474 human breast cancer cells are similar to those obtained with ZD1839, a selective EGFR tyrosine kinase inhibitor which blocks the transactivation of ErbB-2 by the EGFR (Traxler *et al.* 2001, Normanno *et al.* 2002). PKI-166 demonstrated anti-tumor activity in different xenograft models in nude mice (Bruns *et al.* 2000b, Traxler *et al.* 2001). In particular, this compound has been shown to induce apoptosis of endothelial cells and tumor growth inhibition in a human pancreatic carcinoma growing orthotopically in nude mice (Bruns *et al.* 2000b). Furthermore, treatment of mice with PKI-166 was able to inhibit the growth of mouse mammary epithelial HC-11 cells which were infected with a retrovirus encoding the oncogenic NeuT, and subsequently injected in the mammary fat pad of Balb/c syngeneic mice (Brandt *et al.* 2001).

Therapeutic strategies

Although agents directed against the ErbB receptors have shown promising clinical activity, the overall rate of response in cancer patients is generally low. In fact, clinical responses have been observed in a small percentage of patients as compared with the frequency of expression of the target receptors. Cancer is the result of multiple qualitative and/or quantitative abnormalities in molecules that are involved in the regulation of cell proliferation and survival. Therefore, it is conceivable that different growth-promoting pathways might be operating in patients who fail to respond to treatment with single agents directed against the ErbB receptors. In this context, different combinatorial strategies might be employed in order to improve the efficacy of these drugs.

Studies with combinations of anti-ErbB agents and cytotoxic drugs

A way to optimize the efficacy of anti-ErbB therapies is to administer these agents in combination with conventional chemotherapy. In this regard, several studies have demonstrated that anti-ErbB-2 monoclonal antibodies are able to significantly enhance the anti-tumor effects of cisplatin, doxorubicin, thiotepa, etoposide, paclitaxel, methotrexate and

vinblastine (Arteaga *et al.* 1994, Langton *et al.* 1994, Pietras *et al.* 1994, 1998, Baselga *et al.* 1998, Pegram *et al.* 1999). One drug, 5-fluorouracil, was found to be antagonistic with Herceptin *in vitro* and *in vivo* (Pegram *et al.* 1999). Different mechanisms might be involved in this phenomenon. For example, several studies have shown that treatment with anti-ErbB-2 antibodies leads to a significant reduction in the repair of cisplatin-DNA adducts after cisplatin exposure and, as a result, promotes drug-induced killing in target cells (Arteaga *et al.* 1994, Pietras *et al.* 1994, 1998). However, it is not clear whether this phenomenon might be related to ErbB-2 activation, since some of the anti-ErbB-2 antibodies act initially as receptor agonists, but are also able to induce receptor downregulation (Arteaga *et al.* 1994). In this regard, the tyrosine kinase inhibitor CP127,374, which promotes ErbB-2 degradation, impaired DNA repair mechanisms and induced cell accumulation at the G1 phase of the cell cycle in NSCLC cells (You *et al.* 1998). As a possible mechanism of the action of taxanes and anti-ErbB-2 combination therapies, it has been demonstrated that overexpression of ErbB-2 in MDA-MB-435 human breast cancer cells enhances expression of p21Cip1, which associates with and inhibits p34Cdc2 kinase. This phenomenon produces a delay of cell entrance into the G2/M phase of the cell cycle, inhibition of taxol-induced apoptosis and ultimately resistance to taxol (Yu *et al.* 1998a). In this respect, higher expression of ErbB-2 in breast cancer cell lines correlates with resistance to taxol and taxotere (Yu *et al.* 1998b). More recently, it has also been demonstrated that resistance to paclitaxel was associated with a reduced rate of tubulin polymerization in cells that overexpress either ErbB-2 or the mutated EGFRvIII receptor. EGFRvIII-expressing cells demonstrated increases in class IVa and IVb β -tubulin mRNA, and ErbB-2-expressing cells had an increase in class IVa β -tubulin mRNA, suggesting that a correlation between expression of these oncogenes, resistance to paclitaxel and expression of β -tubulin isotypes might exist (Montgomery *et al.* 2000). However, some studies have shown that overexpression of ErbB-2 is not sufficient by itself to induce changes in chemosensitivity in breast and ovarian cancer cells, as well as in human mammary epithelial cells (Pegram *et al.* 1997b, Orr *et al.* 2000). Therefore, alterations in other pathways which regulate cell proliferation and survival might co-operate with ErbB-2 in regulating the sensitivity and/or resistance of tumor cells to chemotherapeutic agents.

Following the results obtained in the preclinical studies, a phase II study of trastuzumab in combination with cisplatin has been conducted in patients with ErbB-2-overexpressing metastatic breast cancer who experienced disease progression during the administration of standard chemotherapy. A 24% response rate was observed in these patients, suggesting that this combination might prove to be extremely effective even in patients refractory to conventional chemotherapy (Pegram *et al.* 1998). These conclusions led to the design of a phase

III trial in patients with advanced breast cancer, over-expressing ErbB-2 (3+ or 2+ as assessed by immunohistochemistry) and unexposed to prior chemotherapy (Slamon *et al.* 2001). In this trial, patients were treated with two different chemotherapeutic regimens (doxorubicin plus cyclophosphamide or paclitaxel) every 3 weeks for six cycles alone or with trastuzumab. The addition of trastuzumab to chemotherapy significantly increased time to progression, response rate, duration of response and overall survival. In particular, a significant difference in overall survival was observed, despite the fact that patients treated with chemotherapy alone at disease progression were crossed over to receive trastuzumab. Although efficacy of trastuzumab was observed in both subgroups, patients with a 3+ score for expression of ErbB-2 benefited to a greater extent from such treatment than patients with 2+ expressing tumors. The major adverse event in patients treated with this combination was cardiac dysfunction, which occurred in 27% of the patients receiving anthracycline plus trastuzumab, and in 13% of the patients receiving trastuzumab plus paclitaxel. In this regard, a recent report has demonstrated that ErbB-2 signaling in cardiomyocytes is essential for the prevention of dilated cardiomyopathy (Crone *et al.* 2002).

In order to further improve the above results, several studies have been conducted with trastuzumab in combination with other chemotherapeutic agents or by using new schedules with taxanes. A phase II study has recently shown that weekly administration of trastuzumab and paclitaxel is active in patients with metastatic breast cancer, with a response rate of up to 81% in patients with ErbB-2-overexpressing tumors (Seidman *et al.* 2001). Although this treatment was generally well tolerated, serious cardiac complications were observed in a small group of patients. A 63% overall response rate was observed in metastatic breast cancer patients treated with weekly trastuzumab and docetaxel (Esteve *et al.* 2002). Interestingly, a good correlation was found between high serum levels of ErbB-2 extracellular domain and response rate in this study. The combination of trastuzumab and vinorelbine seems very promising as well. In fact, a 75% response rate has been recently reported in patients treated with weekly vinorelbine and trastuzumab (Burstein *et al.* 2001). The response rate was even higher (84%) in patients treated with this combination as first-line therapy for metastatic disease, and in patients with ErbB-2 3+ tumors (80%). Finally, recent reports are demonstrating the efficacy of trastuzumab in combination with chemotherapy in tumors other than breast cancer. For example, it has been recently shown that treatment with trastuzumab plus gemcitabine/cisplatin in ErbB-2-overexpressing advanced NSCLC patients is well tolerated with encouraging response rates (Tran *et al.* 2002). Of course, the results of these phase II studies need to be confirmed by phase III studies in which the combination of trastuzumab and chemotherapy are compared with chemotherapy alone.

Several different reports have demonstrated that blockade of the EGFR by either monoclonal antibodies or tyrosine kinase inhibitors can enhance the anti-tumor activity of conventional chemotherapeutic agents. For example, an increased *in vitro* and/or *in vivo* inhibition has been observed when cancer cells have been treated with combinations of C225 plus various cytotoxic agents, including doxorubicin, cisplatin, paclitaxel, gemcitabine and topotecan (Baselga *et al.* 1993, Fan *et al.* 1993, Ciardiello *et al.* 1999, Bruns *et al.* 2000a, Inoue *et al.* 2000). Analogously, increased anti-tumor activity was observed when ZD1839 was combined with cisplatin, carboplatin, oxaliplatin, paclitaxel, docetaxel, doxorubicin, etoposide, topotecan and raltitrexed (Ciardiello *et al.* 2000, Sirotiak *et al.* 2000). However, the mechanisms that are involved in this phenomenon have not yet been clarified. In fact, some reports suggest that overexpression of the EGFR might be associated with resistance to cytotoxic drugs. In particular, increased levels of EGFR have been observed in breast cancer cells that have been selected for multidrug resistance (Dickstein *et al.* 1993, Wosikowski *et al.* 1997). Furthermore, overexpression of EGFR in ZR75B breast cancer cells, which normally express low levels of the receptor, led to increased resistance to several cytotoxic agents, including cisplatin (Dickstein *et al.* 1995). Conversely, several reports have suggested that activation of the EGFR might increase the sensitivity of cancer cells to cytotoxic drugs (Christen *et al.* 1990, Kroning *et al.* 1995). In 2008 ovarian carcinoma cells this mechanism seems not to be dependent upon EGF-induced cell proliferation (Christen *et al.* 1990). However, in ME-180 cervical cancer cells, increased resistance to cisplatin was accompanied by a reduction in the levels of EGFR expression and in the growth rate (Donato *et al.* 2000). Reduction of the expression of the EGFR has also been shown to reduce the sensitivity of MDA-MB-468 human breast cancer cells to cisplatin, without affecting their growth rate (Dixit *et al.* 1997). In this cell line, such a phenomenon is specific for cisplatin, since a similar effect was not seen with other chemotherapeutic agents tested. In this regard, it has been shown that activation of the EGFR reduces the ability of cancer cells to remove DNA adducts following cisplatin treatment (Kroning *et al.* 1995, Dixit *et al.* 1997). Therefore, both activation and blockade of the EGFR pathway seems to have similar effects on the responsiveness to chemotherapeutic agents in different experimental systems. Indeed, these differences might be due to differences in the expression of other elements that are involved in controlling this phenomenon, or of down-stream cascades that are controlled by the EGFR. However, timing of receptor manipulation and exposure to chemotherapy might be critical. In this regard, it has been shown that an additive growth-inhibitory effect occurred when colon carcinoma cells were exposed to EGF-related antisense oligonucleotides after treatment with different concentrations of either 5-fluorouracil, mitomycin C, adriamycin or cisplatin

(De Luca *et al.* 1997). However, treatment of cells with antisense oligonucleotides before exposure to 5-fluorouracil or cisplatin resulted in a reduced efficacy of both drugs in the absence of significant effects on cell cycle distribution. Analogously, the anti-tumor efficacy of a combination of the C225 antibody and topotecan was increased when tumor cells were treated with topotecan first, as compared with concomitant treatment or with pretreatment with C225 (Ciardiello *et al.* 1999). In contrast, a recent report has shown that the effects of ZD1839 with cisplatin and/or 5-fluorouracil were sequence dependent in head and neck cancer cells, with the best results achieved when ZD1839 was applied before and/or during the administration of chemotherapy (Magne *et al.* 2002a). Interestingly, in this report it was found that treatment with ZD1839 after the administration of the chemotherapy might lead to antagonistic effects. It is evident that it is not possible at this point to derive any definitive conclusions from these contrasting results. However, these studies have clearly underlined that blockade of the EGFR might interfere with the activity of anti-tumor drugs.

Following the results of the pre-clinical studies, the feasibility and the activity of combinations of the C225 antibody and cytotoxic drugs in cancer patients have been extensively explored. In a phase I study of C225 in combination with cisplatin, two partial responses (15%) were observed in patients with head and neck cancer (Baselga *et al.* 2000b). This study demonstrated that C225 clearance did not change with the administration of cisplatin. Activity of this combination has also been shown in a phase Ib study in patients with head and neck cancer (Shin *et al.* 2001). In this study, the combination was very effective, with six out of nine (67%) evaluable patients achieving major responses, including two complete responses. A randomized, double-blind, placebo-controlled trial of cisplatin with or without C225 in patients with metastatic/recurrent head and neck cancer has been completed, and survival data will be available shortly. Activity of C225 in combination with cytotoxic drugs has been demonstrated in several different neoplasms. For example, treatment of advanced pancreatic cancer patients with C225 in combination with gemcitabine resulted in anti-tumor activity, with 12% of patients experiencing a partial response and 39% of patients stable disease (Abbruzzese *et al.* 2001). Activity of the combination C225/CPT-11 has been demonstrated in colorectal patients refractory to CPT-11, with a response rate of 22.5% (27/120) (Saltz *et al.* 2001). In this regard, 57 patients have been enrolled in a phase II trial of C225 alone in patients with EGFR positive-colorectal cancer refractory to both 5-fluorouracil and CPT-11 (Saltz *et al.* 2002). Six patients (11%) achieved a partial response. Thirteen additional patients had stable disease or minor responses. Activity of the combination C225 and cisplatin has also been shown in patients with head and neck cancer who were originally refractory to cisplatin (Hong *et al.* 2001).

Pre-clinical studies have shown that C225 enhances the tumor response to ionizing radiation in human epidermoid, head and neck, and colon cancer xenografts (Huang *et al.* 1999, Bianco *et al.* 2000, Huang & Harari 2000, Milas *et al.* 2000). The suggested mechanisms underlying this phenomenon are the accumulation of cancer cells in the G1 and G2-M phases of the cell cycle, the blockade of radiation-induced DNA repair mechanisms, and a reduction of VEGF production in cancer cells (Huang *et al.* 1999, Huang & Harari 2000, Milas *et al.* 2000). These findings have been confirmed by a clinical trial in head and neck cancer patients. Combined treatment of C225 and radiotherapy produced thirteen complete responses and one partial response in a cohort of fifteen patients (Bonner *et al.* 2000). A phase III trial with this combination is currently ongoing in patients with head and neck cancer.

Phase I and II trials of a combination of ZD1839 with several different chemotherapeutic agents are ongoing. A pilot study has shown that ZD1839 in combination with carboplatin/paclitaxel appears feasible and well tolerated in previously untreated patients with advanced NSCLC (Laurie *et al.* 2000). Pharmacokinetic data suggest that ZD1839 does not affect the pharmacokinetics of either carboplatin or paclitaxel. An overall response rate of 25% (six out of twenty-four) has been reported in patients treated with this combination (Miller *et al.* 2001). In an additional phase I trial, chemo-naïve patients with advanced or metastatic solid tumours were treated with oral ZD1839 at two dose levels (250 or 500 mg, once daily) in combination with gemcitabine and cisplatin (Gonzalez-Larriba *et al.* 2002). ZD1839 did not appear to increase the overall toxicity of chemotherapy. Furthermore, ZD1839 had no significant effect on exposure to cisplatin or gemcitabine, except for a small but statistically significant increase in gemcitabine exposure when given with 500 mg/day ZD1839. Of the seventeen patients evaluable for anti-tumour activity, nine had partial responses, seven had stable disease and one progressed. Two large multicenter phase III studies using ZD1839 (250 or 500 mg daily) in combination with cytotoxic agents (carboplatin/paclitaxel or cisplatin/gemcitabine) have been started as first-line treatment in non-operable stage III and stage IV NSCLC patients. Patient accrual for both studies was completed in March 2001. Final data have not yet been presented. However, in August 2002, AstraZeneca announced top-line results of the trials. These results confirmed that the trials were robust and well designed, but demonstrated that ZD1839 does not provide improvement in survival when added to standard platinum-based chemotherapy versus chemotherapy alone in advanced NSCLC patients. These negative data are somehow surprising since in pre-clinical studies it has been shown that ZD1839 improves the efficacy of several anti-tumor drugs (Ciardiello *et al.* 2000, Sirotnak *et al.* 2000). However, as discussed previously, there is no agreement on the optimal sequence of treatment when this combination is employed

(Ciardiello *et al.* 2000, Sirotnak *et al.* 2000, Magne *et al.* 2002a). In this regard, in the phase III trials patients received continuous administration of ZD1839. Therefore, we might hypothesize that such a schedule might interfere with the activity of cytotoxic drugs, therefore annulling the benefits coming from treatment with ZD1839. In particular, we have previously described the potential interactions between EGFR-signaling blockade and sensitivity to cis-platinum.

Finally, preliminary results of a phase I dose-escalation study of daily OSI-774 in combination with gemcitabine and cisplatin have been presented recently (Ratain *et al.* 2002). Three patients were treated in the initial cohort at doses that induced significant neutropenia and nephrotoxicity (gemcitabine, 1000 mg/m² on days 1, 8, 15; cisplatin, 100 mg/m² on day 1; OSI-774, 100 mg on days 1–28, of each 28-day cycle). Furthermore, in one patient a grade 3 increase in the prothrombin time (associated with warfarin) occurred, suggesting that OSI-774 may acutely increase the effects of warfarin through a protein-binding interaction. The protocol was then amended to include a 21-day cycle escalating from a lower dose of cisplatin, and among six patients evaluable for response, two minor responses and two stabilization of disease responses were observed.

Studies with combinations of different anti-ErbB agents

Although combination with conventional chemotherapy represents the simplest way to optimize the efficacy of anti-ErbB therapies, a novel therapeutic approach is taking place. In fact, a common feature of the majority of human carcinomas is that co-expression of different EGF-like growth factors and ErbB receptors frequently occurs. Data also suggest that these molecules co-operate in facilitating the transformation and sustaining the growth of human carcinoma cells. For example, transformation of cells by ErbB-2 requires the co-expression of the EGFR or other ErbB receptors (Cohen *et al.* 1996). Co-expression of ErbB-2 and EGFR is associated with a more aggressive phenotype and/or a worse prognosis as compared with expression of a single oncogene in several tumors such as breast, squamous cell, ovarian and bladder carcinoma and in childhood medulloblastoma (Harris *et al.* 1989, Osaki *et al.* 1992, Simpson *et al.* 1995a, Harlozinska *et al.* 1996, Gilbertson *et al.* 1997, Xia *et al.* 1999, Chow *et al.* 2001). Furthermore, co-expression of different ErbB receptors is associated with increased drug resistance (Chen *et al.* 2000). In this regard, simultaneous blockade of different signal transduction pathways driven by EGF-like growth factors and their receptors might result in a more significant anti-tumor effect as compared with treatment with agents that block a single pathway. Indeed, several reports support this hypothesis. We have recently demonstrated that simultaneous blockade of expression of TGF α , AR and CR in human colon, breast and ovarian cancer cells that co-express these

growth factors, by using combinations of antisense oligonucleotides, results in a more significant inhibition of *in vitro* cell proliferation as compared with treatment with a single antisense oligonucleotide (Normanno *et al.* 1996, De Luca *et al.* 1999, Casamassimi *et al.* 2000). In breast carcinoma cells, a combination of the three antisense oligonucleotides was also able to induce apoptosis (De Luca *et al.* 1999). Similar results were obtained *in vivo* when nude mice bearing human colon carcinoma xenografts were treated with a combination of mixed backbone antisense oligonucleotides directed against TGF α , AR or CR (De Luca *et al.* 2000). Interestingly, a more significant reduction of microvessel count was observed in mice treated with a combination of the three antisense oligonucleotides, as compared with tumors from mice treated with a single oligonucleotide. Furthermore, combination treatment with concurrent exposure to the anti-EGFR antibody C225 and the anti-ErbB-2 antibody 4D5 resulted in additive anti-proliferative effects on human ovarian carcinoma cells, which was accompanied by enhanced G1 cell accumulation, a greater increase in the levels of p27Kip1 and a greater decrease in the activities of CDK kinases (Ye *et al.* 1999). Finally, three different research groups have recently demonstrated that a synergistic growth inhibition occurs when ErbB-2-overexpressing human breast carcinoma cells are treated with a combination of trastuzumab and ZD1839 (Moasser *et al.* 2001, Moulder *et al.* 2001, Normanno *et al.* 2002). Since ZD1839 was shown to block the transactivation of ErbB-2 by either the EGFR or ErbB-3, we might hypothesize that this synergism might be due to the ability of trastuzumab to downregulate ErbB-2 homodimers in ErbB-2-overexpressing tumor cells (Moasser *et al.* 2001, Moulder *et al.* 2001, Anido *et al.* 2002, Normanno *et al.* 2002). Treatment with ZD1839 was also shown to induce apoptosis in breast cancer cells (Moulder *et al.* 2001, Anido *et al.* 2002, Normanno *et al.* 2002). In this regard, trastuzumab did not induce apoptosis but produced an increase of ZD1839-induced apoptosis when breast cancer cells were treated with a combination of the two drugs (Normanno *et al.* 2002). A phase I/II trial of the combination trastuzumab/ZD1839 in breast cancer patients has recently been starting patient accrual.

Conclusions and perspectives

It is clear that the development of anti-ErbB agents might represent a major breakthrough in the therapy of human carcinomas. In fact, clinical responses are seen in heavily pretreated patients. Furthermore, these drugs have a very favorable toxicity profile that allows treatment for long periods in patients who would not otherwise tolerate treatment with cytotoxic drugs. However, several issues still need to be clarified.

For example, with some of these drugs, patient selection criteria have not yet been established. In fact, patients who

have been enrolled in the ZD1839 trials have not been selected for EGFR expression. In this regard, responses have been observed in patients who apparently do not express the EGFR in a phase I clinical trial with OSI-774 (Hidalgo *et al.* 2001). However, no standardized assay to measure the expression of the EGFR has yet been developed. In addition, pre-clinical results suggest that response to anti-EGFR therapies might be observed in tumors with very low levels of EGFR expression which are difficult to detect with routine immunohistochemical techniques. In fact, no correlation has been found in pre-clinical studies between the levels of expression of the EGFR and the response to EGFR tyrosine kinase inhibitors. In this regard, the mechanism of action of some of these agents seems to be more complex than a simple blockade of the target receptor. Specific EGFR tyrosine kinase inhibitors such as ZD1839 have been shown to block the activation of ErbB-2 and ErbB-3 by inducing the formation of inactive dimers of these receptors with the EGFR. Therefore, we can hypothesize that the response to the EGFR tyrosine kinase inhibitors might correlate with the total level of expression of the ErbB receptors in the target cells. The levels of expression of the EGF-related peptides in the tumor and/or in the surrounding stroma might also affect the response to anti-ErbB agents. In fact, the activation of the ErbB receptors and the formation of the different homo- and heterodimers are driven by specific ligands of the EGF family of peptides which are often co-expressed in human carcinomas, as we discussed in the Introduction. A recent report has indeed shown that both the levels of expression of the ErbB receptors and of the EGF-related peptides are able to affect the efficacy of anti-ErbB tyrosine kinase inhibitors (Motoyama *et al.* 2002).

Additional studies are also indeed necessary to select markers of response to the therapy. In fact, although several studies have shown that blockade of the EGFR produces in the patient's skin and/or tumor a significant reduction in the activation of MAPK, no correlation has been established between the efficacy of the therapy and the reduction in the activation of downstream targets such as MAPK or Akt. In fact, some pre-clinical studies have suggested that reduced activation of Akt might represent a marker of sensitivity to EGFR tyrosine kinase inhibitors (Moasser *et al.* 2001). However, other studies failed to find such a correlation (Campiglio *et al.* 2002).

An additional point that needs to be addressed is the possibility of combining anti-ErbB agents and cytotoxic drugs. In fact, recent data from phase III clinical trials with ZD1839 have not confirmed the advantage of such combination in terms of prolongation of survival in NSCLC patients. In this regard, although pre-clinical studies obtained contrasting results, they have clearly pointed out the importance of the sequence in which these agents are administered. The design of the phase II and phase III studies that have been conducted or planned has clearly underscored the possibility that

antagonistic effects between these drugs might occur. Most of these studies are aimed at obtaining the highest response rate. However, this might not represent the ideal target in patients with advanced disease that cannot be cured with currently available treatments. In fact, since the purpose in these patients should be to control the disease for a sustained period, it might be more reasonable to alternate with different drugs. This therapeutic strategy might also avoid the occurrence of antagonistic effects between anti-ErbB drugs and conventional chemotherapeutic agents.

The anti-ErbB drugs might also be combined with other target-based agents. In fact, human carcinogenesis is a complex phenomenon in which alterations of genes other than ErbB receptors and ligands occur. Therefore, combination of anti-ErbB agents with drugs that are directed against other targets that belong to different pathways (cyclooxygenase-2 (COX-2), VEGF receptor, etc) might significantly improve their efficacy. In this context, preliminary findings confirm the feasibility of this approach. For, example, it has been recently reported that combined treatment with COX-2 and HER2/neu inhibitors more effectively reduces colorectal carcinoma growth than either agent alone (Mann *et al.* 2001). Furthermore, a recent study found that combination treatment of APCMin/+ mice (a murine model of human familial adenomatous polyposis (FAP)) with the non-steroidal anti-inflammatory drug sulindac and the EGFR tyrosine kinase inhibitor EKI-569 resulted in complete polyp prevention in half of all treated mice (Torrance *et al.* 2000). Interestingly, mice treated with both EKI-569 and sulindac developed an average number of polyps that was significantly lower as compared with mice treated with a single drug. Since most of these drugs are in clinical studies, we might hypothesize that in the very near future the disease of each tumor patient will be characterized for the expression of these different targets, and each patient will be treated with a different combination of target-based agents and/or cytotoxic drugs. Finally, the possibility of using such combinations for chemoprevention in high-risk patients also needs to be explored.

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