Therapies based on inhibitors of the epidermal growth factor receptor: reaching for the future

REVIEW

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ABSTRACT

The key role played by the epidermal growth factor receptor (EGFR) in different types of solid tumors have turned this molecule into an important target for rational drug design. The contribution of EGFR-related signaling pathways to the promotion of tumorigenic processes, including cell proliferation, angiogenesis, and resistance to apoptosis has been well established. Two classes of anti-EGFR agents currently in late-stage clinical testing include monoclonal antibodies against the extracellular domain of EGFR (Cetuximab, Nimotuzumab) and small-molecule tyrosine kinase inhibitors, which block the enzymatic activity of the receptor (Gefitinib, Erlotinib). In spite of the considerable amount of information gathered from clinical trials with these compounds, important questions such as reliable surrogate markers to predict response to the treatment, or the optimal sequence and combination of these agents with conventional therapies must still be addressed. It has become imperative to identify and validate predictive factors allowing the selection of those patients most likely to respond to EGFR inhibitors, such as mutations that confer resistance versus those associated with sensitivity. A better understanding of the molecular mechanisms associated with antitumoral activity will be useful for predicting the results of the interaction of these agents with traditional therapies, in order to prevent antagonic or redundant effects that do not increase antitumoral activity. Finally, the benefits derived from EGFR inhibitors as first-line therapy in selected populations, and the optimal doses and delivery routes to the tumor site resulting in optimal target modulation should be established by the current research.

Keywords: cancer, epidermal growth factor receptor, monoclonal antibodies, tyrosine kinase inhibitors

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RESUMEN

Terapias con inhibidores del receptor del factor de crecimiento epidérmico: acercando el futuro. La función esencial del receptor del factor de crecimiento epidérmico (EGFR) en diversos tipos de tumores sólidos lo ha convertido en un blanco fundamental para el diseño de nuevas drogas. La contribución de las vías de señalización asociadas al EGFR en procesos tumorales como la proliferación celular, la angiogénesis y la resistencia a apoptosis ha sido bien establecida. Dos tipos de drogas anti-EGFR que están en evaluación clínica incluyen: anticuerpos monoclonales contra el dominio extracelular del EGFR (Cetuximab, Nimotuzumab) e inhibidores tirosina kinasa, capaces de bloquear la actividad enzimática del receptor (Gefitinib, Erlotinib). A pesar de los resultados en pacientes tras la aplicación de estos compuestos, existen aún aspectos que necesitan ser esclarecidos, como la búsqueda de marcadores predictores de buenas respuestas al tratamiento, o las secuencias y combinaciones óptimas de estas drogas con las terapias actuales. Es necesario identificar y validar factores predictivos que permitan seleccionar pacientes que responderán a las terapias con inhibidores del EGFR, como mutaciones que confieren resistencia, o aquellas que incrementan la sensibilidad al tratamiento. Una mejor comprensión de sus mecanismos de acción permitirá predecir la interacción de estos agentes con las terapias actuales, así como prevenir efectos antagónicos o redundantes, que no incrementen la actividad antitumoral. Finalmente, las investigaciones actuales deberán esclarecer las ventajas tras la aplicación de estos compuestos como primera línea de tratamiento, y sus dosis y esquemas de administración óptimos.

Palabras claves: cáncer, receptor del factor de crecimiento epidérmico, anticuerpos monoclonales, inhibidores tirosina kinasa

*I*ntroduction

The epidermal growth factor receptor (EGFR) has been a widely studied molecule due to the key role it plays in the development of many human tumors [1]. EGFR is a 170 kDa membrane glycoprotein composed of three domains: a ligand-binding extracellular domain, a lipophilic transmembrane segment and a cytoplasmic domain with tyrosine kinase activity [2]. EGFR, also known as HER1 and ErbB, is one of the members of the ErbB receptor family, which also includes ErbB2 (Neu, HER2), ErbB3 (HER3) and ErbB4 (HER4); all of them closely related from a structural and functional point of view [1]. This protein plays a very important role in several physiological responses associated with the control of cellular proliferation, differentiation and survival [3-8], and consequently, any alteration of its physiology may lead to the genesis and/or progression of several tumor types, including lung, breast, ovary, pancreas and prostate tumors [1].

Compared to the other members of the ErbB family, EGFR has the unique property of specifically binding at least six different ligands, including, among others, EGF and TGF-alpha. Additionally, EGFR can form heterodimers with other members of this family, thus significantly increasing the complexity of the process

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of receptor activation and the analysis of ligand specificity [9]. These heterodimers are formed preferentially with HER2, a protein with a high oncogenic potential [10, 11], although it is also common to find EGFR homodimers [12], or heterodimers with HER3 [13] or HER4 [14]. In general, the heterodimers (especially those containing HER2) induce signals with a higher biological activity [1].

Not all the details are known about the exact mechanism leading to EGFR activation upon ligand binding, and currently there are two main hypotheses to explain the dimerization process. The first suggests that the ligand binds directly to an EGFR monomer, triggering a subsequent interaction with another monomeric ligand which leads to the formation of a dimer. The second hypothesis proposes that the dimerization occurs with the binding of a ligand to a preexisting receptor dimer, followed by the binding of a second ligand molecule to the complex [10, 12].

The diversity of its ligands, together with the formation of different combinations of homodimers and heterodimers with other ErbB family members which are in turn coupled to different intracellular signaling pathways, illustrates the high complexity of the EGF/EGFR system (Figure 1). The most widely studied, and therefore best characterized intracellular signaling pathway for this system, is that of the Ras/Raf/ERK MAP kinases. Several experimental evidences suggest that many of the biological effects observed after the activation by EGFR ligands may be coupled to the activation of the Ras/Raf/ERK pathway, although these effects are mainly associated to cellular proliferation after the activation by EGF [10]. Another intracellular signaling pathway for activated EGFR is that of phosphatidyl inositol 3-kinase (PI3K), which seems to depend on the levels of protein kinase B (PKB) [15]. Since the effects of PKB are essential for blocking the proapoptotic activities of the Bcl-2 proteins, the EGFR-mediated activation of the PI3K pathway is directly involved in the survival of tumor cells. Other signaling pathways associated to the process of EGFR activation are those of the STAT proteins, phospholipase C-g (PLC-g) and the c-jun N-terminal kinase (JNK); these

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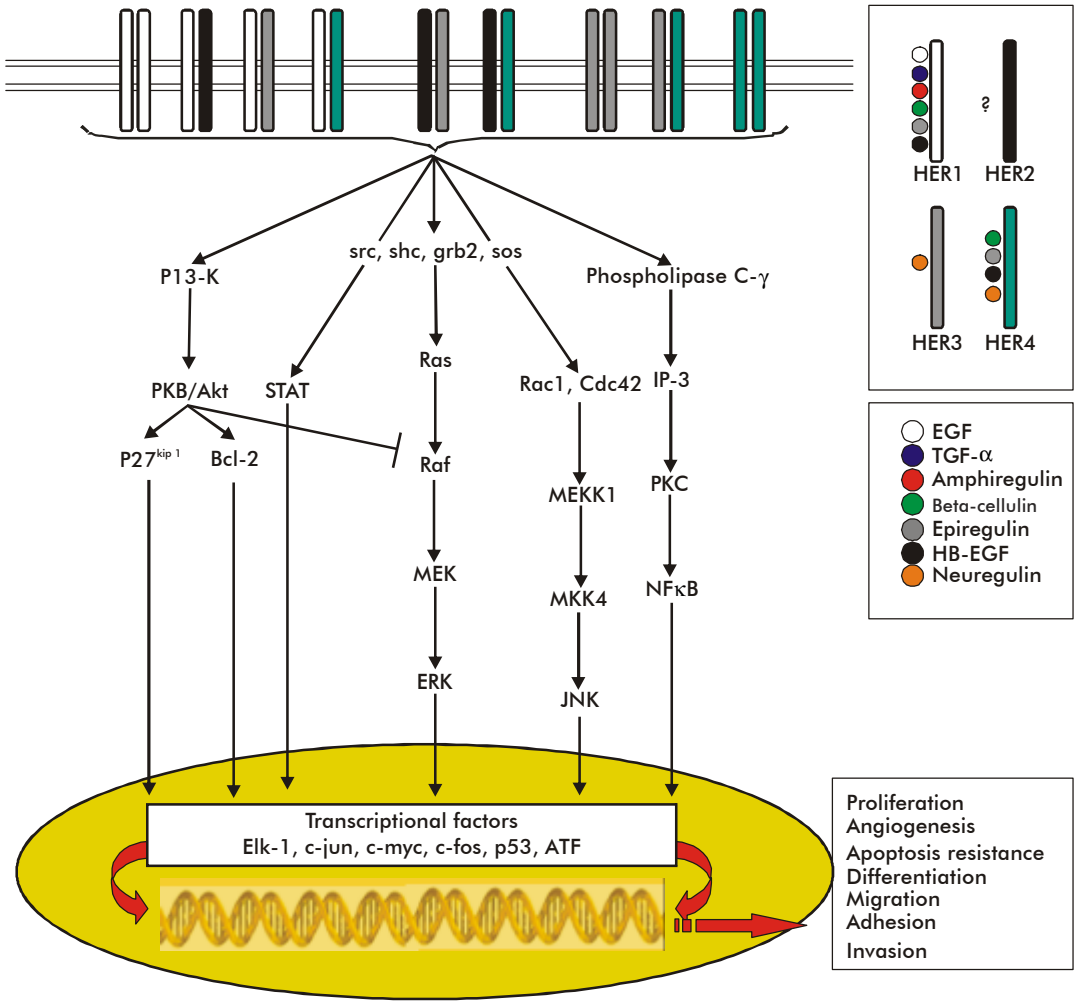

Figure 1. ErbB receptor family and intracellular signaling pathways. After ligand-induced activation, the intracellular residues of EGFR involved in its tyrosine kinase activity can recruit diverse adaptor proteins and signaling molecules. This process triggers complex intracellular signaling cascades that ultimately lead to the transcription of genes that mediate important biological effects at the cellular level.

pathways have been found to be involved mainly in processes of resistance to apoptosis, cell migration, and cell proliferation and transformation, respectively [10].

EGFR is usually expressed at levels ranging from 20 000 to 200 000 molecules per cell, mainly in tissues of epithelial origin. However, in malignant cells these figures can increase to 2 million receptors per cell or higher. A high expression of EGFR is found in several solid tumors, including squamous cell head and neck carcinomas, renal carcinoma, small cell lung carcinoma and colon tumors, among others; this overexpression is caused by several different mechanisms operating at varying levels of the genetic regulation network, e.g. gene amplification, modifications at the transcriptional level, and deletions or mutations that generate constitutively active receptors [16]. It is precisely due to this situation, i.e. the alterations of the physiology of EGFR often turn out to have an oncogenic effect, that the EGF/EGFR system has become a very attractive and promising target for immunotherapeutic interventions based on EGFR-specific antagonists.

Anti-EGFR therapies. Monoclonal antibodies and tyrosine kinase inhibitors

There are currently a number of strategies aimed at the functional inhibition of EGFR, such as the use of tyrosine kinase inhibitors (TKI) [17, 18], toxin-linked monoclonal antibodies [19], antisense oligonucleotides against the EGFR mRNA [20, 21], vaccines [22, 23], and monoclonal antibodies (mAb) specific for the extracellular domain of the receptor or its ligands [24-26]. Within this panoply of alternatives, anti-EGFR mAbs such as Cetuximab (IMC-225; Erbitux) and Nimotuzumab (TheraCIM h-R3), together with TKI such as Gefitinib (ZD1839; IressaTM) and Erlotinib (OSI-774; Tarceva) constitute the most widely studied products, some of which have already been approved for the treatment of patients afflicted by different kinds of tumors.

Both classes of inhibitors (anti-EGFR mAbs and TKI) have proved to be highly effective at blocking signal transduction for MAPK, PI3K/Akt and Jak/STAT [6]. However, even though both strategies are efficient for inhibiting the activation of EGFR and its intracellular signaling pathways, it should be pointed out that they are based on different mechanisms of action (mAbs bind to the extracellular domain of EGFR and block ligand binding, whereas TKI act intracellularly, blocking the binding of ATP to the catalytic domain of the receptor) and this is therefore reflected in the results derived from their direct application (Table 1).

After blocking the activation of EGFR, the anti-EGF mAbs promote the internalization and later degradation of the receptor in the endosomal compartment. This has the net effect of increasing the rate of degradation of this molecule and decreasing the number of signaling-competent receptors on the cellular membrane. In contrast with this situation, TKIs do not remove EGFR molecules from the cell surface, leaving open a chance for EGFR signaling if complete blocking of tyrosine kinase activity is not achieved.

Table 1. Comparison of the main features of monoclonal antibodies vs. tyrosine kinase inhibitors

Monoclonal antibodies	Tyrosine kinase inhibitors
Block the extracellular binding of ligands to EGFR	Compete with ATP for its cytoplasmic binding site on EGFR
Large size (~150 000D) may hinder access to some tumor types	Small size (~400D), allowing better access to some tumor types
The therapeutic activity does not require high concentrations	High concentrations are required for reaching therapeutic activity
High specificity	Specific/ non specific
Low toxicity	High toxicity
They can potentially receive chemical modifications to enhance their therapeutic activity	Available in oral delivery formulations
Induce the internalization and degradation of EGFR at the endosomal level	Do not induce the internalization of EGFR
Acting directly on tumor cells, inducing cell death	Low response levels, used as monotherapy
May induce tumoral resistance	Some mutations may alter the sensitivity to TKIs
May recruit additional immune mechanisms	May overcome tumoral resistance by compensatory mechanisms (non-specific inhibitors)
May potentiate the efficacy of radio and chemotherapies	May potentiate the efficacy of chemotherapy

The high stability and long half life characteristic of mAbs mean that therapeutic efficiency can be reached with only one dose per week, whereas TKI usually require daily doses. On the other hand, mAbs require intravenous delivery, and TKIs offer the advantage of their oral bioavailability. TKIs are small molecular weight compounds which easily reach and are absorbed through the epithelial cells of the intestine, in contrast with mAbs, which are macromolecules and, therefore, might have only very limited access to some anatomical sites of the systemic circulation. This apparent advantage of TKIs, however, may explain the high intestinal toxicity associated to their use.

There are also differences between these EGFR antagonists regarding their specificity and selectivity for inducing receptor blockage. The specificity of mAbs is better than that of TKIs, as is well illustrated by the classification of TKIs into four main groups according to their selectivity and the mechanistic details of their inhibition of tyrosine kinase activity: reversible EGFR inhibitors, irreversible EGFR inhibitors, reversible inhibitors of two members of the ErbB receptor family, and the so-called "pan-ErbB inhibitors", which non-specifically inhibit all members of the ErbB family [27]. However, this lower specificity can be advantageous in certain settings, e.g. the mAbs may be unable to inhibit signaling in tumors where EGFR forms heterodimers with other ErbB family members, and in this situation TKIs (especially those active against two or more ErbB family members) stand a better chance for success. Likewise, while an effective mAb-based therapy requires the presence of an intact ligand-binding domain, this is not the case for TKIs, which can still be active in the presence of mutated EGFR receptor forms. These theoretical advantages of TKIs, however, have not been translated into better experimental results; for example, Gefitinib was not very effective at inhibiting NR6M tumors that overexpressed EGFRvIII [28] (EGFRvIII is a mutated variant of EGFR with a deletion on the extracellular domain of EGFR that spans exons 2 to 7 [29]), whereas Cetuximab has proved to be able to bind the extracellular domain of EGFRvIII in glioma cells [30].

Lastly, mAbs –especially those of the IgG1 isotype

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such as Cetuximab and Nimotuzumab- may be able to activate other immunological defense mechanisms, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) through the binding of immune effector cells to their Fc domains [31].

In short, each of these strategies has different characteristics that define their potential to benefit cancer patients; therefore predicting which of them will be most effective during actual treatment is not an easy task.

Cetuximab

Cetuximab is a monoclonal antibody which exclusively binds the extracellular domain of EGFR, effectively blocking the binding of its ligands. This molecule was initially developed as a murine antibody and was later chimerized with a human IgG1 in order to decrease the chances of generation of a human immune response against murine antibodies (HAMA). The chimerization increased the affinity of Cetuximab for EGFR by about one order of magnitude, up to a Kd of 10^{-10} M from that of 10^{-9} M showed by the natural ligand (EGF) and the original murine mAb 225 [32]. Cetuximab has proved to be potently inhibitory for the proliferation of the A431 epidermoid carcinoma, both *in vitro* and in tumors grafted in athymic mice; it also has been shown to have an important synergistic effect with the activity of cytotoxic drugs and radiotherapy. These results led to the implementation of clinical trials to further study this mAb. The first trials recruited patients affected with solid tumors having high expression levels of EGFR, including colorectal carcinoma, non small-cell lung carcinoma, and head and neck squamous cell carcinomas; the results showed that the mAb, whether administered as a monotherapy or in combination with cytotoxic drugs or radiotherapy, was highly efficient. However, a significant increase of the toxicity induced by conventional therapies was also observed. The most common side effects associated to the use of Cetuximab include fever, asthenia and nausea after a prolonged period of administration, although more severe effects may also appear such as an acneiform rash and even anaphylactic shock in up to 2% of the treated patients [33]. It should be noted, though, that the side effects associated to skin toxicity are reversible and not limiting for the use of this molecule at the required therapeutic dose [34]. Cetuximab constitutes the first monoclonal antibody antagonist for EGFR licensed by the Food and Drug Administration to be used in human patients. It was first registered as a monotherapy or in combination with Irinotecan for the treatment of colorectal carcinoma patients with detectable EGFR expression, in late stages of the disease [35]; and has recently been registered also for the treatment of head and neck tumors in combination with radiotherapy. Among the first clinical results obtained with Cetuximab (which led to its approval for the treatment of colon cancer) were a randomized Phase II trial that included 329 patients with disease progression after the treatment with Irinotecan [36], which evaluated the antitumoral response, the progression rate of the disease, the survival and the side effects of the antibody, either as a monotherapy or in combination with Irinotecan. The combined Cetuximab/Irinotecan treatment resulted in partial or total responses in 22.5% of the treated patients, although survival did not increase significantly. Although side effects were more frequent in the group treated with the combination regime, their incidence and severity were not higher than expected in the group treated with Irinotecan monotherapy alone. Other trials have included patients treated with Cetuximab combinated with Irinotecan, Fluorouracil and Leucovorin [37, 38], reporting in all cases important levels of antitumoral response and a good tolerance to the combination.

Cetuximab has also been used in squamous cell head and neck carcinomas, with remarkable success when combined with radiotherapy in patients at locoregionally advanced stages of the disease. In an international multicenter trial that recruited 424 patients, the addition of Cetuximab to radiotherapy practically doubled the mean survival time of the subjects (from 28 to 54 months), while also increasing the percentage of surviving patients after two and three years under treatment from 55 and 44% respectively for the patients under radiotherapy only to 62 and 57% for those receiving the combination therapy [39].

Nimotuzumab

The humanized mAb Nimotuzumab (TheraCIM h-R3) is, like Cetuximab, an antibody specific for the extracellular domain of EGFR. Although both antibodies have similar mechanisms of action, they also have some important differences [40]: Nimotuzumab has a lower affinity for EGFR (10^{-9} M) than Cetuximab (10^{-10} M), which might explain the lower toxicity associated to the side effects affecting the skin for this mAb. In this sense, it is generally agreed that there is an optimum affinity range for compounds acting as EGFR antagonists; and this has been used to explain why agents such as Nimotuzumab, whose affinity for EGFR is not so high, have an optimal biological activity at doses considerably below the toxic threshold. Another important difference between these antibodies is their source: whereas Nimotuzumab was developed by humanizing a murine antibody obtained by the previous immunization of mice with human placenta enriched with EGFR [41], Cetuximab is a chimeric antibody developed from a murine antibody obtained from a cell line.

Nimotuzumab, similarly to Cetuximab, has shown a potent antitumoral effect both *in vitro* and in preclinical models, based on its antiproliferative, antiangiogenic and proapoptotic characteristics [42]. Among the main clinical results obtained for Nimotuzumab is a Phase I/II trial that included 24 patients with advanced head and neck squamous cell carcinomas, who received a combined treatment with radiotherapy and different doses of the antibody [40]. According to the results, the objective response index was 87.5% (14 out of 16 patients), and there was a complete response in 68.75% of the cases (11 out of 16 patients) in those subjects treated with doses of 200 and 400 mg of the antibody. Likewise, the mean survival time for the patients treated with these doses also increased significantly (44.30 months, compared with 8.60 months for the patients receiving doses of 50 and 100 mg). The antibody-radiotherapy combination was well tolerated, and most of the toxic

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effects were associated with the larger doses of the antibody; this toxicity was classified as medium or moderate, since there was only one event of grade III toxicity. The complete absence of any cutaneous rash in all the treated patients was also significant and worth noting.

Another relevant clinical result is the use of Nimotuzumab in combination with radiotherapy for the treatment of malignant gliomas. A report recently published by Crombet *et al*. [43] about a Phase I/II multicenter trial which included 29 patients with glioblastomas or anaplastic astrocytomas was the first publication dealing with the results of such a combination in this type of patient. The treatment reached a level of objective response of 37.9% (17.2% complete response, and 20.7% partial response), and in 41.4% of the patients the disease remained stable. Furthermore, a relative increase in the mean survival time after a 29 month follow-up was also reported, with a value of 17.47 months for the patients afflicted with glioblastomas (the figures were not yet available for the astrocytoma patients at the date of the publication). The trial also revealed that the use of Nimotuzumab did not increase the toxic effects associated with the radiotherapy, and there were neither grade 3 or 4 side effects, nor acneiform rashes or allergic reactions. For the sake of comparison, previous studies dealing with the use of TKIs in glioblastoma patients did not report objective tumoral responses in groups of 53 and 19 subjects treated with Gefitinib or Erlotinib, respectively [44, 45].

Gefitinib

In contrast with the anti-EGFR mAb strategy, the use of TKIs in clinical settings has not yielded the expected results. These compounds have failed to exhibit, during the course of clinical trials, the antitumoral activity that was anticipated on the basis of their significant antitumoral effects *in vitro* or in preclinical models. There have been no significant antitumor effects for TKIs in glioblastoma or colon cancer patients; the most promising, albeit modest results, have been obtained mainly in non-small cell lung carcinomas [46].

Two multicenter clinical trials, known as IDEAL 1 and IDEAL 2 (IressaTM Dose Evaluation in Advanced Lung cancer), evaluated the biological activity of Gefitinib at different dosages (250 and 500 mg) in a total of 452 patients with advanced non-small cell lung carcinoma which were previously refractory to chemotherapy [47, 48]. These two trials, implemented in parallel by independent groups, did not show significant differences between the two doses as judged by the antitumoral response (18.4 vs. 19%), control of disease progression (40.3 vs. 37%), or survival. However, there was treatment-associated dose-dependent toxicity, evidenced by an increased frequency and severity of side effects. The recommendation derived from this trial was, therefore, the use of 250 mg doses daily for subsequent trials.

There have been two large randomized Phase III clinical trials to evaluate the efficacy of Gefitinib as a monotherapy or in combination with chemotherapy as a first-line treatment for the management of non-small cell lung carcinomas. These trials, named INTACT 1 and INTACT 2 (Iressa NSCLC Trial Assessing Combination Treatment), recruited 1 093 and 1 037 patients, respectively. The results were not encouraging and served as fodder for a number of interpretations, some of which will be analyzed later on. INTACT 1 evaluated the efficacy of Gefitinib in combination with Gemcitabine and Cisplatin, comparing the results to those for patients receiving only chemotherapy plus a placebo [49]; on the other hand, INTACT 2 evaluated a combination of Gefitinib with Paclitaxel and Carboplatin, also comparing the results to those of patients receiving only the chemotherapy plus a placebo [50]. None of the trials showed differences, as evaluated by survival or control of disease progression, between the Gefitinib/chemotherapy combination and chemotherapy alone (average survival for INTACT1: 10.9, 9.9 and 9.9 months for the groups receiving placebo, Gefitinib 250 mg and Gefitinib 500 mg, respectively; average survival for INTACT 2: 9.9, 9.8 and 8.7 months for the groups receiving placebo, Gefitinib 250 mg and Gefitinib 500 mg).

There was also another trial known as ISEL (Iressa Survival Evaluation in Lung cancer) designed to evaluate the effect of Gefitinib on survival when used as second- and third-line treatment for patients with advanced non-small cell lung carcinoma which had been refractory to Irinotecan or other chemotherapies [51]. This Phase III trial recruited 1 692 patients from centers located in 28 countries from Asia, Europe, Australia, South America, the U.S. and Canada. The results did not show significant differences as evaluated by the objective response (complete or partial) between Gefitinib and the placebo group (8% vs. 1%); there were no differences, either, in survival: The average survival (5.6 months) and the survival after a year (27%) of the patients treated with Gefitinib did not differ from those of patients receiving only the placebo (5.1 months and 21%). All these results have led researchers to question seriously the validity of the use of TKI for the treatment of non-small cell lung carcinomas [52].

Erlotinib

Erlotinib, like Gefitinib and other TKIs, has been evaluated mainly in non-small cell lung carcinoma. An a priori comparison of the main properties of Erlotinib with those of Gefitinib is not so favorable to the former, and the clinical results reached by Erlotinib can, at most, be classified as discrete. The maximum tolerated dose (MTD) of Erlotinib has been determined, based on previous Phase I clinical trials, to be 150 mg per day [53]. A first Phase II trial that recruited 57 patients with non-small cell lung carcinomas which were refractory to previous chemotherapy yielded a response to Erlotinib of 12%, with 40% of the patients surviving for one year [54].

In another trial named TRIBUTE (Tarceva Response in Conjunction with Taxol and Carboplatin), 1 059 patients received Erlotinib or a placebo combined with Carboplatin and Paclitaxel as a first-line treatment for advanced non-small cell lung carcinoma [55]. Erlotinib in combination with chemotherapy did not offer a better survival time (10.6 months) when compared to chemotherapy alone (10.5 months), and there were no

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significant differences between both treatments concerning the progression rate of the disease, the levels of objective response and the length of the response.

A trial called TALENT (TArceva Lung cancEr iNvestigaTion), which was conducted outside the United States, recruited 1 172 patients to receive Erlotinib or placebo combinated with Gemcitabine or Cisplatin [56]. Like the other trials above, this one failed to detect significant differences between the treatments.

Finally, a randomized Phase III study implemented by the National Cancer Institute of Canada (BR21; 731 patients), comparing the efficacy of Erlotinib between patients that had previously progressed while under chemotherapy and patients receiving only placebo and medical care, did find an increase in the levels of objective response (8.9% vs. less than 1%) and survival (6.7 months vs. 4.7 months), favorable to Erlotinib [57]. These results led to the approval by FDA of Erlotinib as second- and third-line treatment for advanced stage non-small cell lung carcinoma patients.

Optimization of anti-EFGR compound-based therapies

Molecular targets and predictors of response

Unlike traditional cytotoxic therapies such as chemo and radiotherapy, the current strategies target a well-defined molecular entity and hence require a high degree of precision. Obviously, a detailed knowledge of the main molecular factors affecting tumor biology is needed to ensure that all relevant targets are considered during treatment design. This need for the optimization of immunotherapeutic interventions has led to the search for reliable markers that can be used for the early prediction of patient evolution and can become the basis for the prospective selection of those individuals most likely to be benefited by a specific therapy. The first potential marker examined as a predictor of efficacy for treatments based on EGFR activity blockers was precisely the receptor itself; perhaps due to previous experiences showing that the efficacy of Herceptin and Tamoxifen depends to a great extent on the expression levels of the molecules targeted by these drugs [58].

The results of the studies designed to examine the relevance of EGFR as a prognostic factor –regardless the treatment in the evaluation of cancer patients- have generated considerable debate and varying opinions within the scientific community. The expression levels of EGFR can be very different from one tumor type to another, and this is further complicated by differences in the detection methods used by different research groups. The percentage of EGFR-positive tumors in some types of solid tumors, such as non-small cell lung carcinomas, can vary within a range of 40 to 80%, even reaching 100% in the case of head-and-neck tumors [59]. Other solid tumors, such as melanomas, have significantly lower levels of EGFR, to the point that the positivity criteria themselves become controversial [60, 61].

In some tumor types, such as those of squamous cells of the head and neck, the expression of EFGR seems to be strongly correlated to increases in the recurrence of the disease, a reduction in patient survival, progress to advanced stages of the disease and an increased appearance of metastases. Furthermore, there are reports for breast cancer patients that associate the expression of EFGR with a significant increase of the proliferative index and a reduction in survival [62]. These results have led researchers to consider the expression levels of EGFR as an important predictive factor in these tumors, whose significance even overshadows that of other markers. However, this criterion has not been solid enough for its application to other tumors, such as lung and colon carcinomas. There is an excellent review by Nicholson [16] which analyzes the relationship between the expression of EGFR, relapse-free interval and patient survival in ten different tumor types, across more than 200 studies totaling more than 20 000 patients. This analysis showed that the prognostic value of the EFGR expression levels can vary significantly between different tumor types. For instance, whereas this parameter was a good predictor of patient outlook for head and neck, ovarian, bladder and esophageal cancers, its predictive value was only modest for other malignancies such as breast, gastric, colorectal and endometrial cancers. Even more discouraging were the results for non-small cell lung tumors, where the expression levels of EGFR were only rarely related to patient outlook.

In antitumoral drugs that block the activity of EGFR, it is logical to expect a higher efficiency in tumors expressing this protein at high levels, while their effect should decline (or even disappear) in tumors that are negative for EGFR. However, it should be pointed out that defining a tumor as receptor-negative does not mean that this protein is completely absent, since the current immunohistochemical methods classify a tumor as receptor-positive only if there are more than 30 000 receptor molecules per cell. For example, the assumption above has determined that Cetuximab, which has been approved for the treatment of colorectal carcinomas, should be indicated only for EGFR-positive tumors as classified by the positivity criterion of the EGFR pharmDx kit (manufactured by DakoCytomation and approved by the FDA) [63]. However, the results of the Phase II trial that used Cetuximab combined with Irinotecan in patients with chemotherapy-refractory colorectal carcinoma showed that treatment efficacy did not correlate well with EGFR expression levels [64]. Similar results have been obtained from the application of Gefitinib in patients suffering from breast cancer [65] or advanced stage non-small cell lung carcinomas [54]. The idea that many tumors could be treated similarly –as if being clinically and biologically identical- is now being abandoned, and the scientific community is accepting a very different reality: the levels of expression of the EGFR protein may be necessary, but are insufficient to account for the success or failure of EGFR inhibitors. This reality partially explains the results obtained from the application of some EGFR antagonists, as illustrated by non-small cell lung tumors. The clinical results accumulated during the use of Gefitinib against them indicate a better response in adenocarcinomas than in squamous cell tumors, even though the

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expression levels of EGFR are significantly lower in the former (44%) than in the latter (82%) [66]. The interpretations derived from these analyses might lead, on the one hand, to the search for new EGFR-associated targets (either proteins or signaling pathways), but on the other they may also lead to the premature detention of research on some of these drugs due to the lack of positive results.

The potential response predictors that have recently become of interest are the basal level of activation of EGFR and the dependence of the intracellular signaling pathways of the receptor. One example, still in preclinical evaluation, is the detection of mutated forms of the PTEN protein phosphatase that is resistant to TKIs. Certain researchers have pointed out that while Gefitinib inhibited the phosphorylation of EGFR in the MDA-468 breast cancer cell line at concentrations as small as 0.1 mM, it required concentrations higher than 1 mM to inhibit signaling via the PI-3K/Akt pathway. This resistance was associated with the presence of a mutated form of the PTEN protein [67]. Even more interesting is the study published by Li *et al*. [68], showing that Gefitinib is unable to inhibit the activation of Akt and ERK in malignant glioma lines, even though it successfully blocks the activation of EGFR and STAT-3. In this paper, the authors concluded that it is impossible to effectively inhibit Akt- and ERK-mediated signaling with doses lower than 2 to 5 mM; however, the dosing schemes for this drug currently in clinical use prescribe doses of 250 mg/day, which are equivalent to a plasmatic concentration of only 0.45 mM [69]. Gefitinib has not had good antitumoral activity in some preclinical studies, particularly those involving this type of tumor [28].

Another predictor that is being intensely studied is the presence of dimers and mutated forms of EGFR. It has been established that the expression of HER2 may potentiate EGFR signaling [70] and contribute to tumoral transformation and progression [71]. An example of this are the synergistic effects of the combination of Cetuximab with anti-HER2 antibodies in ovarian tumors with high HER2 expression [72]; or the potentiation of the antitumoral effect of Trastuzumab when combined with Gefitinib for the treatment of xenografted BT-474 breast tumors with high gene amplification of HER2 [73]. The expression of HER3 has been associated with a positive response to TKI-based therapies. In advanced stage lung cancer patients receiving treatment with Gefitinib, it was possible to observe that a high copy number of the HER3 gene was associated with a higher response level (36 vs. 10%) and a longer time to disease progression (7.7 vs. 2.7 months), although not with increased survival (10 vs. 11 months) [74]. Additionally, the presence of somatic mutations in the catalytic domain of EGFR has also been proposed as a relevant marker for the prediction of a positive response to TKI treatment [75]. However, this idea is currently under debate, given the low frequency of these mutations in important groups of the population, and the recent finding of secondary mutations in lung cancer patients treated with TKI that developed tumor resistance to these drugs [76].

The cutaneous rash associated with EGFR blockage has been another important potential predictor for the treatment with EGFR inhibitory compounds. Several authors have associated this rash to signaling inhibition on skin cells, ultimately leading to an arrest of the cell cycle in keratinocytes and an increased level of the p27Kip1 protein [77]. However, several contradictory results have cast doubt on the hypothesis of the skin rash as a reliable predictor for the response to receptor-blocking agents. A study published by Saltz *et al*. [64] shows that there is rash in 96% of the patients with good responses to a combined Cetuximab/Irinotecan treatment; however, in this same study, 74% of the patients that did not respond to the treatment had similar allergic reactions. On the other hand, the significant levels of response to the Nimotuzumab antibody do not seem to be associated with the development of a cutaneous rash on the treated patients [40, 43]. This apparent contradiction might partly be a consequence of the different affinities of both antibodies for EGFR. As previously explained, Nimotuzumab may block EFGR effectively in tumors without inducing adverse events in skin cells. In this sense, it is interesting to compare the optimal doses originally proposed for the treatment of patients with Cetuximab on the basis of preclinical mice studies with the much higher doses required to achieve saturation, which were verified in Phase I trials [78]. This difference is not observed for Nimotuzumab, even though both inhibitors share the characteristic of binding human but not murine EGFR; and it suggests that, unlike Nimotuzumab, Cetuximab may exert a significant blockage on non-tumor cells expressing EGFR.

Other important factors when trying to maximize success during the optimization of this antitumoral therapy are related to the dosage schemes used for these compounds. Unlike conventional therapies, where the maximum allowed dose depends almost solely on the toxicity of the drug or therapy to be used, therapies with anti-EGFR immunotherapeutic compounds require the definition of the optimal biological dose and of the treatment schemes that achieve a complete and sustained saturation and/or inhibition of EGFR activity [79]. Examples given earlier in this review prove the counterintuitive hypothesis that the optimal therapeutic doses for some of these compounds might not match the doses needed for an optimal biological activity [68]. Similarly, the optimal treatment sequence required for EGFR inhibitor therapies will only be defined by experience and the thorough analysis of results of clinical trials. So far, these compounds have generally been evaluated in patients with metastases or at very advanced stages of the disease, after previous cytotoxic treatments. In the future, it will become necessary to change this situation and evaluate these agents as first-line treatment options, either in monotherapy or in combination with traditional cytotoxic therapies. Experimental evidence shows that this might be the correct strategy: the overexpression of EGFR in hyperplasic and neoplastic lesions suggests that this may occur years before the appearance of the invasive metastatic

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expression of the disease [80]; and some of these compounds have worked better as first-line therapy than as second- or third-line therapies on patients with previous therapeutic failures from conventional cytotoxic treatments [81].

Combination therapies

The facts detailed in the previous section explain the cause of the modest results after the administration of EGFR inhibitors to cancer patients, due in part to the absence of reliable markers to predict the response to this treatment. This idea is further reinforced by the marked patient-to-patient variability which is typical of these therapies where, while some patients respond very effectively to the treatment, others have very poor or practically no response levels. There is a second hypothesis, which does not exclude the former consideration, that is based on the redundancy characterizing the control methods of the immune system over tumoral cells. This hypothesis suggests that the control of the proliferation of tumor cells might be resistant to immunotherapeutic interventions aimed at a single point, with the corollary that therapeutic combinations will be needed to increase the efficacy of the antitumoral effects of these treatments.

For a cell to acquire a malignant phenotype, a number of changes or alterations must have taken place that lead to its transformation into a tumor cell [82]. The end result of this process is the alteration of a set of diverse and redundant signaling pathways, and therefore the combination of different drugs with dissimilar mechanisms of action may be necessary to achieve the modulation level of a specific pathway. This premise has set the rules for the search and application of rational therapeutic combinations based on the current therapies. Many of the results shown above suggest that the treatments based on the inhibition of EGFR may be combined, with favorable results, with cytotoxic drugs, ionizing radiations, cytokines and other agents intended for the treatment of tumors expressing EGFR at high levels.

The observations revealing that mAbs and TKIs have complementary mechanisms of action, together with the fact that both classes of inhibitors bind different (non-competitive) sites on EGFR, indicate the possibility of combining these drugs to potentiate the antitumoral effect that they both exhibit separately. This attractive idea is now being tested, and although the combinations are still in preclinical evaluation, the initial results are encouraging [83]. Furthermore, there are, numerous preclinical studies proving that the inhibition of EGFR, whether via mAbs or TKIs, can potentiate the activity of cytotoxic drugs or radiotherapy in cell lines sensitive to receptor inhibitors. The radiosensitization mediated by anti-EGFR compounds may be produced, at least in part, by alterations in the cellular cycle, the inhibition of DNA damage repair mechanisms by the radiation, or the elimination of survival signals essential for the cell in a situation of cell cycle arrest, all of which lead to apoptosis [84]. The addition of (mainly) monoclonal antibodies to radiation therapies has proved to be superior to traditional ionizing radiation-based treatments administered as a monotherapy [39].

The joint application of molecular compounds inhibiting EGFR and cytotoxic drugs such as Carboplatin, Paclitaxel and Gemcitabine, is perhaps the setting where most clinical experience has been gathered. Although some results have not been as successful as expected, for many the combination therapies are still superior to the traditional cytotoxic treatments. This statement is based on the proven capacity of EGFR-inhibiting compounds of reverting the resistance generated by traditional therapies [54, 57, 85].

The combination of EGFR antagonists with drugs acting intracellularly to inhibit relevant, specific molecular targets such as the MAPK or Akt proteins clearly has potential for raising their antitumoral activity. The activation of EGFR leads to an increase in the transcription of proteins such as the receptor for vascular endothelial growth factor (VEGFR). Therefore, the inhibition of EGFR can modulate the expression of these proteins and facilitate the action of drugs acting directly on these targets. A recent example of this situation is the study published by Lamszus *et al*. [86], where the inhibition of angiogenesis is potentiated by the joint application of the anti-EGFR mAb C225 and an anti-VEGFR-2 (type 2 VEGF receptor) antibody. Likewise, an increase has been found in the resistance of A431 cells (an epidermoid carcinoma with high EGFR expression, sensitive to mAb-mediated EGFR inhibition) to treatment with the Cetuximab and Nimotuzumab antibodies, which can be traced back to an augmented expression and secretion of VEGF *in vivo* [87]. These results indicate, therefore, that a combined therapy with anti-EGFR agents together with angiogenesis inhibitors might cut both ways, simultaneously increasing the efficacy of both drug types and thus potentiating the overall antitumoral effect.

Although it has not been determined whether cancer patients do or do not have a limited response capacity to a treatment with a mAb that also stimulates the ADCC response, this might be a fundamental premise for the administration of mAbs in combination with immunopotentiating cytokines such as interleukin 2 (IL-2), which stimulate immune effector functions. This possibility may be vitally important, specially for the treatment of older patients, or in advanced stages of the disease, or subjected to rigorous cytotoxic treatments such as chemotherapy or radiotherapy that result in a status of significant immunosuppression.

Conclusions

The therapies to inhibit tumor growth based on the inhibition of EGFR with molecular agents represent a new opportunity for the successful treatment of cancer patients (Table 2). These treatments seem to be substantially superior to the conventional alternatives employed so far. Some of these agents are under evaluation in patients, and the search and selection of appropriate markers that allow an early prediction of the tumor types that will most likely be controlled by a specific therapy is now in the hands of preclinical research teams and depend on the evaluation derived from clinical trials. This will increase the benefits of

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the therapy for the patients and will, at the same time, minimize or eliminate unnecessary exposures to therapies which are often invasive. The rational combination of these molecular agents with the traditional cytotoxic therapies may lead to a higher status quo in cancer treatment, where the possibility of observing tumoral remissions or arresting disease progression finally becomes a reality, and it becomes feasible to use prolonged low-toxicity treatments that may extend patient survival with an appropriate quality of life.

Table 2. Summary of the main reasons supporting the status of EGFR as a relevant target in cancer therapy

Number	EGFR: a relevant target for cancer treatment
1	EGFR is a protein TK involved in the process of cellular signaling
2	EGFR is the product of an oncogene
3	EGFR is expressed in many different tumor types at levels considerably higher than those found in normal tissue
4	EGFR overexpression seems to be associated with a bad prognosis and an adverse evolution of the disease in some tumor types
5	Several immunotherapeutic agents specific for EGFR inhibit its biological activity, which leads to important antitumoral effects.

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