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Anti-EGFR monoclonal antibody in cancer treatment: *in vitro* and *in vivo* evidence

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1. ABSTRACT

The complexity of EGFR signaling network suggests that the receptor could be promising targets for new personalised therapy. In clinical practice two strategies targeting the receptor are available; they utilise monoclonal antibodies, directed towards the extracellular domain of EGFR, and small molecule tyrosine kinase inhibitors, which bind the catalytic kinase domain of the receptor. In this review, we summarise currently known pre-clinical data on the antitumor effects of monoclonal antibodies, which bind to EGFR in its inactive configuration, competing for ligand binding and thereby blocking ligand-induced EGFR tyrosine kinase activation. As a consequence of treatment, key EGFR-dependent intracellular signals in cancer cells are affected. Data explaining the mechanisms of action of anti-EGFR monoclonal antibodies, currently used in clinical setting and under development for the treatment of solid tumors, are revised with the aim to provide an overview of the most important preclinical studies showing the impact of this class of EGFR targeted agents on tumor biology.

2. INTRODUCTION

The epidermal growth factor receptor EGFR also known as ErbB-1/HER1 is one of the four known members of the ErbB family of tyrosine kinase receptors, (RTKs) including ErbB-2 (neu, HER2), ErbB-3 (HER3) and ErbB-4(HER4) (1,2). These trans-membrane proteins play a fundamental role in the communication between outside and inside cellular compartments transducing external signals through the membrane into the cell.

All ErbB proteins share four functional domains: an extracellular ligand-binding domain, a single hydrophobic transmembrane domain, an intracellular tyrosine kinase domain and a C-terminal regulatory domain (3).

In particular, the extracellular domain is divided into four subdomains and it is the less conserved domain among receptors, suggesting different specificity in ligand binding (4). The tyrosine kinase domain is formed by an N-lobe and a C-lobe and between these lobes ATP takes place; the C-terminal regulatory domain encompasses many domains that are phosphorylated after ligand binding; the intracellular tyrosine kinase domain is the highly conserved one (5).

EGFR activation occurs through the binding to receptor monomers of ligand, such as the epidermal growth factor (EGF), the transforming growth factor alpha (TGFα) and so on, which promotes dimer formation and enhances catalytic activity through tyrosine auto-phosphorylation. After ligand binding, EGFR can form EGFR-EGFR homodimers as well as heterodimers with other members of the ErbB receptor family (6, 7, 8).

The activation of EGFR by ligands determines the phosphorylation of several tyrosine residues, within the kinase domain, which serve as doking sites for SH2-containing signaling proteins capable of transducing intracellular signals.

Beside ligand dependent phosphorylation of EGFR, receptor activation can also occur after treatment with unphysiological stimuli, including hyperosmolarity, oxidative stress, mechanical stress, UV light and gamma-irradiation. This effect is due to the inactivation of phosphatases that antagonize the intrinsic receptor kinase activity, promoting the activated state (9).

The activation of EGFR triggers at least five different signaling pathways: that of mitogen-activated protein kinase (MAPK), phospholipase C, the phosphatidylinositol 3-kinases (PI3K) / Akt, JAK / STAT, and SRC/ FAK , leading to increased proliferation, resistance to pro-apoptotic stimulus, and production and release of pro-angiogenic factors (4,10-12).

EGFR signalling pathways are tightly controlled in normal cells conversely, in tumor cells, aberrant expression of EGFR, activating mutations in it and/or overexpression of receptor ligands lead to enhanced receptor activity contributing to tumorigenesis (13).

A brief description of pathways dependent on the activation of EGFR is shown below.

2.1. MAPK Signaling Pathway

The MAPK signalling pathway play a pivotal role in transducing EGFR mediated signalling (14). After EGFR activation and tyrosine kinase phosphorylation, GRB2/Sos complex adaptor proteins, through Shc adaptor protein, binds to specific intracellular EGFR docking sites (15), inducing a structural changes of Sos, with the recruitment of Ras-GDP and consequent Ras activation (Ras-GTP), which in turn activates RAF1, MAPK1 and MAPK2 (MK01 and MK03) (16). Activated MAPKs phosphorylate and regulate the expression/activity of specific intranuclear transcription factors, ultimately involved in the control of cell proliferation, differentiation, survival and migration (17).

2.2. Phospholipase C signaling pathway

Phosphoinositide signaling involving PLCgamma is under EGFR control. Ligand-binding to EGFR induces the dimerization of the receptor with its subsequent phosphorlation and activation through conformational change. It is this change responsible for the binding to PLCgamma. Hydrolysis of phosphoinositol 4,5 bisphosphate (PIP2) induces the production of diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3). The last one, through the binding to the IP3 Receptor on the endoplasmic reticulum, releases Ca2+ into the cytoplasm, which together with DAG, can activate various PKC isoforms. Ca2+ also binds to various calcium-binding proteins such as calmodulin. The calcium-mediated results include changes in the cytoskeleton and in cell adhesion thus, this pathway is mainly involved in the regulation of tumor invasion and migration (18).

2.3. PI3K/Akt Signaling Pathway

EGFR-dependent activation of PI3K occurs following the dimerization of EGFR with ErbB-3 (8). PI3Ks are dimeric enzymes composed of a catalytic (p110) subunit and a regulatory one (p85). This latter subunit is directly associated by mean of its Src homology domain 2 (SH2) to receptor-specific docking sites (19). p110 subunits catalyze the phosphorylation of phosphatidylinositol 4,5-diphosphate to the second-messenger phosphatidylinositol 3,4,5-triphosphate which in turn phophorylates and activates the protein serine/threonine kinase Akt (20). The serine-threonine kinase Akt, phosphorylates and regulates the activity of a number of cellular mediators including kinases, transcription factors and other regulatory molecules. Through phosphorylation of these targets, Akt carries out its role as a key regulator of a variety of critical cell functions including glucose metabolism, cell proliferation and survival (21).

2.4. STAT signaling pathway

Another pathway controlled by EGFR is the one that originates with the activation of STAT by phosphorylation. Tyrosine kinases that phosphorylate STAT are activated by the interactions between cytokines or growth factors and their respective receptors, which a consequent receptor dimerization or oligomerization.

Cytokine receptors without intrinsic kinase activity need Janus kinases (JAKs) to induce STAT receptor docking and activation conversely, EGFR can activate directly STAT proteins. In its activated for STAT forms dimers which ultimately translocate to the nucleus, where they bind to DNA inducing transcription of specific target genes (22). In tumor tissues, however, constitutive activation of STAT ultimately leads to increased cell proliferation, cell survival, angiogenesis, and immune system evasion (23).

2.5. SRC/ FAK signaling pathway

Src family of kinases (SFKs), are transducers of mitogenic signaling emanating from several RTKs like EGFR, fibroblast growth factor receptor (FGFR) and platelet derived growth factor (PDFGR) (24). The interaction between Src and membrane receptors results in enhancing receptor dependent tumorigenic effects, and in inducing resistance to RTKs targeted agents (25). The association of SrcC with the plasma membrane, through its myristoylated SH4 domain leads to the autophosphorylation of Tyr419 enabled by interactions with activated receptor tyrosine kinases (26, 27). Src dependent activation of FAK induces the phosphorylation of substrates such as paxillin, CAS and p190 RHOGAP bringing about changes in the cytoskeleton which lead to focal-adhesion disruption (28, 29). Activation of FAK stimulates also the c-JUN aminoterminal kinase (JNK) signaling pathway, ultimately leading to increased expression of the matrix metalloproteinases MMP2 and MMP9 which promote the breakdown of the extracellular matrix (ECM) required for tumour invasion of surrounding tissues (30). Src activation leads to STAT activation which induces the increased expression of vascular endothelial growth factor (VEGF), a signalling molecule that promotes tumour angiogenesis (31, 32).

2.6. ADCC: antibody-dependent cellular cytotoxicity

In recent years, several reports have shown that the anti-tumoral effects of anti-EGFR MoAb may be due to their ability to act on the immune system. These antibodies are able to elicit antibody-dependent cellular cytotoxicity (ADCC). ADCC can be viewed as a mechanism to directly induce a variable degree of immediate tumor destruction that leads to antigen presentation and the induction of tumor-directed T-cell responses. ADCC is due to the binding of the Fc portion of antibodies to the Fc receptors expressed on the surface of different cell types, and to the specificity of the Fc receptors for a given Ig class. Different cell population express specific Fc receptors, such as neutrophils which express human FcgammaRI (CD64), FcgammaRII (CD32) and the B (lipid-anchored) isoform of FcgammaRIII (CD16) and the human natural killer (NK) cells which have only CD16. The binding of the antibody to the Fc receptor induces the release of cytokines, such as IFN-gamma, and cytotoxic granules, containing perforin and granzymes, which at the end stimulate apoptosis (33, 34).

The complexity of EGFR signaling network suggests that the receptor, as well as other down stream effectors, could be promising targets for new personalised therapy, using drugs acting on specific cell mediators. For what concerns EGFR inhibitors, there are several potential strategies targeting the receptor; among them, monoclonal antibodies (MoAbs), directed towards the extracellular domain of EGFR, and small molecule tyrosine kinase inhibitors, that interfere with receptor signalling by targeting its catalytic kinase domain, are currently in clinical practice (35).

In this review, we summarise currently known pre-clinical data on the antitumor effects of recombinant, human/mouse chimeric monoclonal antibodies (MoAbs) that bind specifically to the extracellular domain of EGFR, thereby inducing the inhibition of its signalling. Revised data are related to biological properties attributed to MoAbs, such as i) the direct inhibition of EGFR tyrosine kinase activity, of cell cycle progression and of angiogenesis, invasion and metastatization processes, ii) the activation of pro-apoptotic molecules and iii) the induction of cytotoxic effect through antibody-dependent cellular cytotoxicity (ADCC).

3. CETUXIMAB

3.1. Mechanism of action

Several MoAbs have been developed against EGFR. The first mouse monoclonal antibodies specific for human epidermal growth factor (EGF) receptors have been prepared using as immunogen protein from human A431, epidermoid carcinoma cells. One of the most active among these antibody was the 225 (36). However, murine proteins are associated with the development of human antimouse antibodies (HAMA) which cause an increased risk of allergic reactions decreasing the efficacy of the MoAbs thus the 225 was chimerized (37). The murine sequences (about 34% of total antibody) was added with human IgG1 constant region to avoid human antimouse antibody production and to increase its clinical utility (37, 38).

This human/murine chimeric antibody directed against the EGFR is Cetuximab (IMC-C225, ErbituxTM) (39). It has a molecular weight (MW) of 152-kDa and is composed by four polypeptide chains: two identical heavy (gamma) chains and two identical light (kappa) chains held together by covalent and non-covalent bonds and consisting of 449 and 214 amino acids, respectively (40).

Cetuximab has shown considerable activity towards several solid tumours and was approved by Food and Drugs Administration (FDA) in February 2004 for use as single agent or in combination with chemotherapy in the treatment of metastatic colorectal cancer (mCRC), in combination with radiotherapy (RT) for locally/regionally advanced head and neck squamous cell carcinoma (HNSCC), and as monotherapy for recurrent/metastatic HNSCC after failing platinum-based chemotherapy (39, 40).

However, cetuximab therapy is associated with a severe side effect, skin rash, due to immunogenic reaction because it contains murine sequences (41).

Cetuximab acts by binding extracellular region of EGFR. Crystallographic studies enlighten the structural basis of inhibition of this receptor

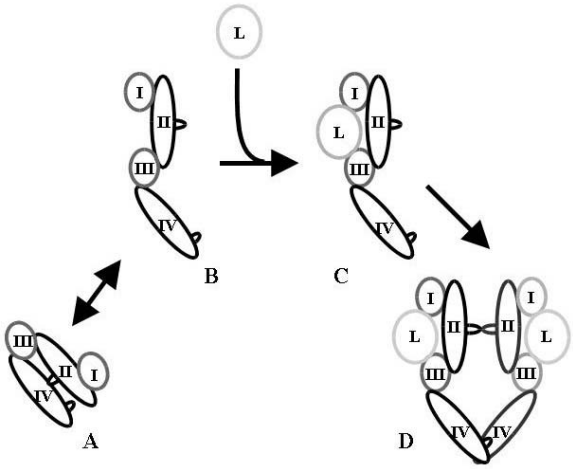

Figure 1. Mechanism of ligand-induced EGFR dimerization. The domains I-IV of the sEGFR are shown in picture and ligand is indicated by L. (A) Tethered monomers; (B) untethered monomers; (C) extended conformation stabilized by ligand; (D) activated dimers induced by ligand

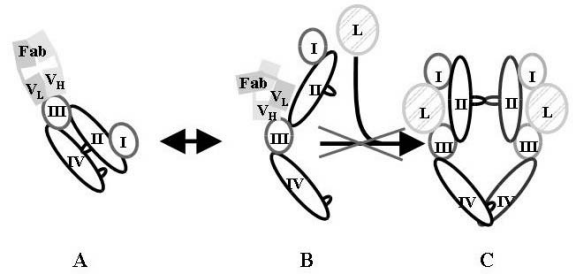

Figure 2. Model for inhibition of ligand-induced dimerization by cetuximab. The domains I-IV of the sEGFR are shown in picture and ligand is indicated by L. VH and VL are heavy and the light chains, respectively

The soluble extracellular region of EGFR (sEGFR) contains four domains (numbered from I to IV). This receptor exists at the cell surface in two different affinity classes: with 2%–5% of receptors binding EGF with high affinity (K_D < 0.1 nM) and 92%–95% binding with lower affinity (K_D 6–12 nM). The classes of affinity are due to different possible receptor conformations (Figure 1) (42).

About 95% of the unbound EGFR is present in a compact conformation stabilized by intramolecular interaction between domains II and IV (or tether). The receptor in this conformation is inactive (A), but in the remaining 5% of the unbound EGFR, this interaction is broken, and the sEGFR can assume a range of untethered conformations (B).

Ligand binds preferentially the latter conformation, interacting simultaneously with domains I and III, stabilizing the extended form and exposing the domain II (C) (43). This region of domain II is known as "dimerization arm", it is rich in the cysteine-residues and provides the majority of interactions with other monomers causing receptor dimerization (D) (44).

Growth factors, as EGF or TGF-alpha, drive the equilibrium shown in Figure 1 to the right, binding preferentially to the untethered or extended forms of EGFR (Figure 1B) and blocking the receptor in this conformation that can dimerize (Figure 1C) (42, 43).

This mechanism of action suggests different ways for an inhibitor to interact with the extracellular region of EGFR preventing its activation: i) it could act as an antagonist (competing directly for ligand binding); ii) it could block ligand binding indirectly (stabilizing an inactive receptor conformation); iii) it could block receptor dimerization and activation by occluding the domain II (43).

Cetuximab binds exclusively to domain III of tethered state of sEGFR with both the heavy (V_H) and the light (V_L) chains changing the distribution of sEGFR conformations that are accessible (Figure 2). Neither the structure of the antibody nor the structure of domain III change upon binding. Moreover, the V_H region of the antibody sterically blocks domain I and domain II which cannot adopt the conformation required for dimerization.

Whereas binding of growth factor ligand requires both domains I and III of sEGFR, cetuximab blocks growth factor binding through high-affinity interaction with domain III alone and acts as a competitive inhibitor (43, 45). Therefore, the receptor cannot bind ligand with high-affinity and cannot dimerize (43).

This antibody targets EGFR with higher affinity compared to TGF-alpha or EGF and it is capable to promote EGFR endocytosis, intracellular trafficking and receptor downregulation (23, 46).

Yoshida *et al.* investigated the effects of cetuximab in human non small cell lung cancer (NSCLC) H292 cells, which express wild-type EGFR. They showed that this antibody is able to induce EGFR phosphorylation by activating the tyrosine kinase of the receptor, and this effect is completely blocked by gefitinib, a small molecule inhibitor of EGFR. Moreover, the antibody induces EGFR dimerization (47); this effect is agree with its immunologically bivalent binding capacity, as shown for MoAb 225 by Fan *et al.* (48). The complex antibody-EGFR remains at the cell surface and does not induce endocytic trafficking and degradation. This is responsible for the failure activation of downstream signaling by Akt and Erk1/2 signal transduction pathways (47).

Similar results have been provided by Mandic *et al.* the treatment of head and neck squamous cell carcinomas (HNSCCs) with cetuximab led to EGFR activation due to hyperphosphorylation of tyrosine, but inhibits Erk1/2 phosphorylation (49).

3.2. Biological properties

Direct inhibition of EGFR activation is considered the main mechanism for the antitumor activity

of cetuximab *in vivo* however, also others, not yet completely investigated, are involved in determining its effectiveness (43). They include the inhibition of cell cycle, the increase and activation of pro-apoptotic molecules, the inhibition of angiogenesis, invasion and metastatization (50-56). Moreover, cetuximab can determine antibody-dependent cellular cytotoxicity (ADCC) that could contribute to its antitumoural effect (57, 58).

3.2.1. Inhibition of cell cycle progression and induction of apoptosis

Cell cycle progression from G_1 to S phase is controlled by the activation of cyclin-dependent kinases (CDKs), which are in turn controlled by interactions with other proteins, including cyclins and the CDKIs. Progression into S-phase is controlled, also in part, by the availability of growth factors.

Wu *et al.* investigated the effect of cetuximab on cell cycle progression in DiFi human colon adenocarcinoma cells and they showed a G_1 arrest induced by EGFR blockade, which is associated with the inhibition of CDK2 activity and induction of the CDKI $p27^{KIP1}$ (50).

Analogue results have been found by Peng *et al.*: cetuximab inhibits proliferation of androgen-independent DU145 prostatic cancer cells by arresting cell cycle progression in G_1. This cell line is retinoblastoma (Rb) deficient and the G_1 arrest induced by the antibody is not followed by apoptosis conversely, in DiFi cells, in which Rb is expressed, cell cycle regulation is followed by programmed cell death (51).

Further studies to deepen mechanisms responsible for apoptosis induction by cetuximab have been carried out. They demonstrated that in DiFi cells addition of insulin-like growth factor-1 (IGF-1) or high concentrations of insulin to cetuximab can delay apoptosis induced by antibody, acting through the IGF-1 receptor conversely, G_1 arrest was unaffected. This suggests that the regulation of cell cycle progression and that of apoptosis are distinct and that common pathway(s) activated by both EGF receptors and IGF-1 receptors may be involved in inhibition of apoptosis in these cells (52).

Also Liu *et al.* investigated how IGF-1 and basic fibroblast growth factor (bFGF) modulate G_1 arrest and apoptosis induced by cetuximab in DiFi cells. Both bFGF and IGF-1 activated the MAPK/MEK pathway additionally, IGF-1 activated the PI3K/Akt pathway. Both bFGF and IGF-1 inhibited cetuximab-induced apoptosis however, bFGF reversed this effect with an increase in the $p27^{Kip1}$ level, inhibition of CDK-2 activity, dephosphorylation of the Rb protein and the consequent G_1 arrest of the cells, suggesting the involvement of the MEK/MAPK pathway. Conversely, IGF-1 delayed the onset of apoptosis, but it did not prevent cell cycle arrest. This mechanism is mainly mediated by the PI3K/Akt pathway (53).

Other studies about apoptosis have been conducted by Scablas *et al.*. They found that in MDA Panc-28, a human pancreatic tumor cell line, the treatment with cetuximab leads to a marked decrease in constitutive NF-kappaB DNA binding activity and to downregulation of *bcl-xl* and *bfl-1*, two antiapoptotics members of the bcl-2 family. This result agrees with other studies that show that EGFR can activate NF-kappaB in various human cancer cell lines. Moreover, when cetuximab is given with gemcitabine there is a significant increase in apoptosis induced by downregulation of antiapoptotic genes (54).

3.2.2. Inhibition of angiogenesis, invasion and metastatization

Metastasis represents a multistep process that requires migration, invasion, and angiogenesis. Angiogenesis is regulated by balance between pro-angiogenic and antiangiogenic factors.

Several studies show that cetuximab can modulate the expression of angiogenic growth factors such as vascular endothelial growth factor (VEGF), bFGF and interleukin-8 (IL-8).

An *in vitro* model of angiogenesis uses HUVEC cells, human umbilical vascular endothelial cell line, because when plated, these cells are capable of forming tube-like structures. Cetuximab can reduce cell-to-cell interaction, resulting in disruption of tube formation.

In *in vivo* studies, using tumour xenograft neovascularization model of angiogenesis, showed that treatment with cetuximab reduces tumour growth, number of blood capillaries, and inhibits the growth of vessels toward the tumour (55).

Other studies *in vitro* about angiogenesis have been conducted by Perrotte *et al.*, using cetuximab in human transitional cell carcinoma (TCC) of the bladder cell line 253J B-V.

They observed an inhibition of mRNA and protein production of VEGF, IL-8 and bFGF by cells; they evaluated also the potential for this agent to inhibit angiogenesis *in vivo*, using nude mice with established orthotopic TCCs. This is resulted in the down-regulation of these angiogenic factors and consequently in the involution of blood vessels (56).

Huang *et al.* examined the effect of molecular blockade of EGFR on the invasive and metastatic capacity of human squamous cell carcinoma (SCC) of the head and neck using *in vitro* and *in vivo* model systems. They observed that treatment with cetuximab attenuated the migration of SCC-1 tumour cells through a chemotaxis chamber in a dose-dependent manner. Using an *in vivo* orthotopic xenograft model, locoregional tumour invasion of SCC-1 into muscle, vessel, bone, and perineural tissues was inhibited in cetuximab-treated mice.

Tumour invasion and metastasis are characterised by deregulated proteolysis and by release a series of extra-cellular proteinases such as MMPs. Cetuximab has been shown to inhibit the expression and

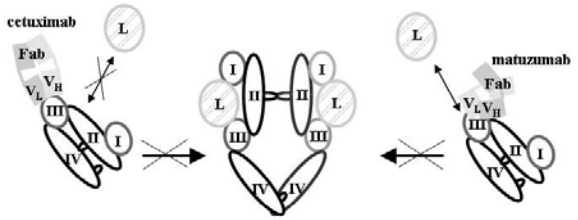

Figure 3. Model for inhibition of ligand-induced dimerization by matuzumab. Matuzumab blocks ligand-induced EGFR dimerization and activation with a mechanism different from that utilized by cetuximab. The domains I-IV of the sEGFR are shown in picture and ligand is indicated by L.

activity of several MMPs including the gelatinase MMP-9, both *in vitro* and *in vivo* (55).

3.2.3. ADCC: antibody-dependent cellular cytotoxicity

Cetuximab is an IgG1, for this reason is able to mediate ADCC induced by NK activity through binding to FcgammaRIII however, it is also able to engage Fc receptors on the surface of other cells such as eosinophils, mast cells, dendritic cells, B cells and other cell types (57).

Kurai *et al.* investigated the ADCC activity of cetuximab against non–small-cell lung cancer (NSCLC) cell lines. They showed that cetuximab was capable of activating ADCC activity efficiently even against lung cancer cells, which weakly express EGFR, and that NK cells were primarily responsible for the cetuximab-mediated ADCC activity and the augmentation by IL-2 (58).

4. MATUZUMAB

4.1. Mechanism of action

Matuzumab (EMD 72000) is a humanized IgG1, anti-EGFR antibody derived from murine MoAb 425, produced by immunization of mice with human A431 epidermoid carcinoma cells (59). The ‘‘humanization’’ of the antibodies tries to keep only the complementarity determining regions (CDRs) of rodent sequences (about 10% of total antibody); that substitution removes much of the immunogenic reactions caused from chimeric antibodies, like cetuximab (37).

Actually, matuzumab is in phase II clinical trials, in combination with chemotherapy in in esophago-gastric cancer and advanced lung cancer (59) [www.clinicaltrials.gov].

This antibody inhibits EGFR activation binding to domain III of the extracellular region of EGFR (sEGFR) and sterically blocking *a)* the conformational changes in domain II and the formation of critical contacts with domain III, interactions required for stabilize the dimerization, and *b)* preventing domain I from reaching the position required for ligand binding with high-affinity (Figure 3).

This is a noncompetitive mechanism, in fact the access to the ligand-binding site on domain III is not blocked. This mechanism is different from that described for cetuximab, which is a competitive inhibitor; in fact, both antibodies block EGF binding to EGFRs, but they interact with distinct epitopes of domain III. This allow the possibility for the two antibodies to simultaneously bind the receptor enhancing its internalization and degradation, leading to a synergistic antitumor effects (59).

4.2. Biological properties

4.2.1. Inhibition of cell cycle progression and induction of apoptosis

Yoshida *et al.* investigated the effects of matuzumab in human NSCLC H292 cells showing that this antibody, like cetuximab, is able to induce EGFR phosphorylation and dimerization, to inhibit receptor turnover and the activation of the downstream Akt and Erk1/2 signaling pathways (47).

Other *in vitro* studies have been conducted by Meira *et al.* to investigate molecular mechanisms that underlay the cytotoxic effects of matuzumab towards human squamous carcinoma A431 cells and to compare them to cetuximab mechanism of action. They found that treatment with matuzumab does not decrease A431 cell viability and induces only a slight increase (0,4%) of cells accumulation in G_0/G_1 phase of the cell cycle. This antibody efficiently binds EGFR and blocks Akt phosphorylation, but is not able to inhibit MAPK cascade. Instead, cetuximab is more efficient in inhibiting A431 cell proliferation and cell cycle arrest; its mechanism of action is similar to that of matuzumab with the addition of the inhibition of Erk1/2 phosphorylation. When given together, matuzumab and cetuximab synergistically reduced the number and size of A431 colonies, induced EGFR down-regulation and reduced Erk1/2 and Akt phosphorylation. The simultaneous blockage of both MAPK and Akt pathways may explain the synergistic effects obtained by this combination (41).

Kleespies *et al.* investigated the effect of matuzumab in the human pancreatic cancer cell line L3.6pl, *in vitro* and *in vivo*. They showed that this antibody inhibits EGFR, MAPK and Akt phosphorilation both *in vitro* and *in vivo* with a significant antiproliferative activity. The inhibition of MAPK pathway is amplified when matuzumab is given in combination with gemcitabine; they inhibit proliferation, induce tumor cell apoptosis and cell cycle arrest and accumulation of cells in G_0/G_1 phase (60).

4.2.2. Inhibition of angiogenesis, invasion and metastatization

Kleespies *et al.* demonstrated that matuzumab inhibits cells migration *in vitro* and *in vivo,* in an orthotopic nude mice model. This antibody showed marked antimetastatic activity, inhibiting lymph node and liver metastases and reducing microvessel density. Also these effects are enhanced by the addition of gemcitabine (60).

Meira *et al.* reported that cetuximab and matuzumab, given together, efficiently decreased VEGF mRNA expression, suggesting their potential role in inhibition of angiogenesis *in vivo* (41).

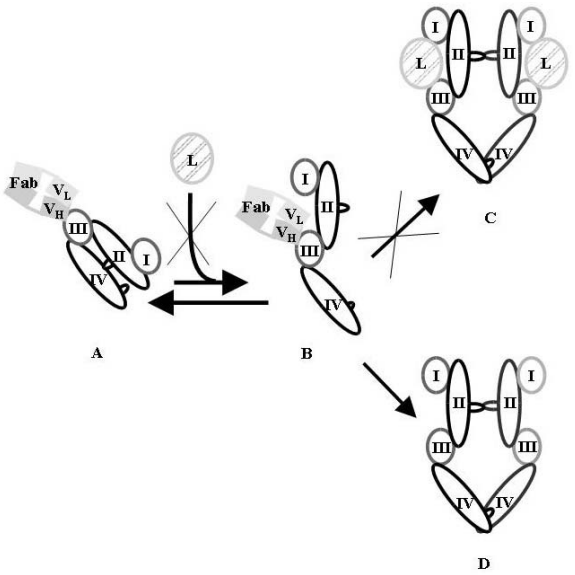

Figure 4. Model for inhibition of ligand-induced dimerization by nimotuzumab. Nimotuzumab is able to maintain the existing balance between the tethered (A) and extended EGFR conformation (B); it inhibits EGF binding and blocks EGFR ligand-dependent dimerization (C) but it does not interfere with basal EGFR ligand-independent dimerization (D). The domains I-IV of the EGFR are shown in picture and ligand is indicated by L.

4.2.3. ADCC: antibody-dependent cellular cytotoxicity

Matuzumab is an IgG1 and is able to generate antitumor ADCC in squamous cell carcinoma lines of head and neck (SCCHN) (61). Moreover, cetuximab and matuzumab can induce ADCC *in vitro* in A431 cells and in several cell lines (41).

Recently, the ADCC activity of matuzumab was investigated in an *in vivo* model. When the antibody was enzymatically de-glycosylated *in vivo*, its ADCC activity is significantly reduced if compared to the native antibody (62).

5. NIMOTUZUMAB

5.1. Mechanism of action

Nimotuzumab, previously known as h-R3, is a humanized IgG1 monoclonal antibody against human EGFR (63). It was generated from its murine counterpart, m-R3, by "grafting" the complementary determining regions (CDRs) of the murine IgG2 to an IgG1 human framework, which reduces its immunogenicity (64). This antibody is in phase III clinical trials for the treatment of advanced nasopharyngeal cancer in combination with cysplatin and radiotherapy, glioblastoma multiforma and head & neck cancer (63) [www.clinicaltrials.gov].

Nimotuzumab binds to domain III of the extracellular region of the EGFR by the heavy chain and it approaches domain I with variable light chain in the superimposed EGFR structures, without clashing into it. Nimotuzumab sterically inhibits EGF binding while it allows EGFR domain I to approach domain III so the receptor can adopt the extended, active conformation. The epitope recognized by nimotuzumab strongly overlaps with the cetuximab binding site; the first antibody binds EGFR with a dissociation costant (K_D) 2.1 x 10^{-8} M, whereas the second has K_D = 1.8 x 10^{-9} M. Moreover, nimotuzumab is able to maintain the existing balance between the tethered and extended EGFR conformation and it doesn't interfere with the basal level of EGFR signaling, which is necessary for the survival of normal epithelial cells (Figure 4). These mechanisms and the intermediate affinity of nimotuzumab, compared with other anti-EGFR antibodies, can explain the low degree of adverse effects and the low toxicity observed in the clinics (65).

5.2. Biological properties

5.2.1. Inhibition of cell cycle progression

Crombet-Ramos *et al.* analyzed the growth-inhibitory potential of nimotuzumab *in vitro* in 2-dimensional (monolayer) and 3-dimensional (spheroid) cultures of A431 cells. They found a maximum antiproliferative activity of 40% when cells are treated *in vitro* for 48 hr, this effect is similar in either monolayer or spheroid cultures. Moreover they showed, with the analysis of DNA profile, a G_1 arrest in treated A431 cells, accompanied by a decrease in the S phase. Therefore, they concluded that this antibody acts mainly as a cytostatic rather than as a cytotoxic agent *in vitro*, and that is confirmed from the absence of an apparent hypo-diploid peak, representative of apoptosis (66). Nimotuzumab has levels of pro-apoptotic and antiproliferative activity in A431 colonies equivalent to cetuximab, at their most effective doses (67).

5.2.2. Inhibition of angiogenesis and induction of apoptosis

The activation of EGFR by ligand exerts a proangiogenic effect by inducing vascular endothelial growth factor (VEGF) expression.

Crombet-Ramos *et al.* found that blockade of EGFR activity can result in downregulation of VEGF expression *in vitro* and *in vivo*. In fact, they demonstrated that nimotuzumab inhibits VEGF production in A431 monolayer cultures in a dose dependent manner and significantly reduced VEGF mRNA expression of A431 tumors growing subcutaneously in SCID mice (64, 66, 68). Conversely, they showed that this antibody did not have any effect on the levels of bFGF protein, another angiogenic factor influenced by the EGFR, secreted by these cells.

Moreover, Crombet-Ramos *et al.* found *in vivo* reduction of the overall microvascular density (MVD), and of the caliber of detectable vascular channel. They supposed that this antiangiogenic activity of nimotuzumab *in vivo* is often followed by apoptosis of the cancer cells themselves, in fact they found five-folds increase in the apoptotic index of the antibody-treated tumors, this latter is indicative of a cytotoxic mode of action *in vivo*. In addition, they observed an antiproliferative effect of this antibody *in vivo*, with significant decrease in the mitotic activity of treated vs. control tumors. That was associated with reduction in Ki67-

positive tumor (a marker of cell proliferation). These results suggest that the anticancer properties of nimotuzumab are associated with combined and potent antiproliferative, antiangiogenic and proapoptotic activity (66).

5.2.3. ADCC: antibody-dependent cellular cytotoxicity

Nimotuzumab can promote antineoplastic effect with another mechanism operating *in vivo* but not in cell culture: the activation of immunologic effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) (64, 66). Nevertheless, this mechanism of tumor regression seems less probable (66).

6. IMC-11F8

6.1. Mechanism of action

IMC-11F8 is a fully human anti-EGFR antibody (69). It is characterized by the absence of murine proteins and by the reduction of immunogenic reactions (37). This antibody is in phase I clinical trials (63).

IMC-11F8 has a mechanism of action similar to cetuximab, in fact it binds exclusively to domain III of tethered state of sEGFR and sterically blocks domain II which cannot adopt the conformation required for dimerization. Both antibodies recognize the same EGFRs epitope on domain III and the binding affinity for this receptor is very similar, nevertheless the interactions made by IMC-11F8 are quite different.

Cetuximab contains murine sequences that can cause immune hypersensitivity in clinical trials; this problem is surpassed with IMC-11F8 that shows similar antitumor potency as cetuximab and less side-effect (70).

6.2. Biological properties

Meilin Liu *et al.* investigated the effect of IMC-11F8 in several tumor cell lines and they observed a block of EGFR and MAPK phosphorylation. This antibody also inhibits the *in vitro* growth of different tumor cell lines EGFR-overexpressing, such as DiFi (colorectal), A431 (epidermal) and BxPC3 (pancreatic) [Proc Amer Assoc Cancer Res, Volume 45, Abstract #706, 2004, unpublished data].

Further *in vivo* studies have been conducted by Prewett *et al.*. They evaluated the activity of antibody in models of epidermoid (A431), pancreatic (BxPC-3) or colon (HT-29 and DLD-1) human tumor xenografts in athymic mice. They observed an inhibition of tumor growth dose-dependent in all mice models; in addition they evaluated the effect of IMC-11F8 in combination with irinotecan in colon xenograft models (DLD-1, GEO, or HT-29) which resulted in synergistic anti-tumor effect in all three tumor models [Proc Amer Assoc Cancer Res, Volume 45, Abstract #5353, 2004, unpublished data].

7. ZALUTUMUMAB

7.1 .Mechanism of action

Zalutumumab, previously known as HuMax-EGFr, is a fully human IgG1κ monoclonal antibody against human EGFR (63). This antibody is obtained by transgenic mice in which the gene clusters encoding murine Ab H and L chain genes have been inactivated and DNA segments have been introduced containing large parts of the human H and (kappa) L chain gene clusters. Then, these mice were immunized with human target proteins and they produce high amount of Abs (71). Being this antibody completely human it has a low risk of hypersensitivity reactions (72).

Zalutumumab is in phase II clinical trials for the head and neck squamous cancer cell treatment (63).

This antibody binds EGF-binding site on domain III and inhibits EGFR ligand binding. Even cetuximab binds to domain III and there is a cross-block with zalutumumab, indicating that the two antibodies compete for binding. Further studies demonstrated that epitopes are not identical but overlap (73).

Zalutumumab inhibits EGFR activation via two distinct mechanisms. When it binds domain III, it prevents EGFR from adopting the extend and active conformation. This first mechanism resembles that described for cetuximab and is based on the monovalent interaction of the antibody Fab arm with the EGFR protein. The second mechanism is when zalutumumab binds bivalently the receptor and inactives it, forming dimers by spatial separation of two molecules of EGFR (73).

7.2. Biological properties

Zalutumumab has dual mode of action in function of drug concentration. At high concentration with a full receptor saturation, it completely prevented receptor phosphorylation in overexpressing EGFR A431 cells, blocking EGFR signaling and inhibiting cell *in vitro* proliferation (71). This antibody has the same effect in HN5, MDA-MB-468, and BxPC-3 cells with different efficiency correlated to the difference in autocrine EGF production and/or the level of EGFR overexpression (71). Instead, a second anti-tumor effect is present at much lower concentrations, when the receptor is partially saturated. Zalutumumab induced antibody-dependent cell cytotoxicity (ADCC) *in vitro* mediated by mononuclear (MNC) and polymorphonuclear (PMN) cells (74) [Journal of Clinical Oncology, 2006 ASCO Annual Meeting Proceedings (Post-Meeting Edition) Vol 24, No 18S (June 20 Supplement), 2006: 2543, unpublished data].

Bleeker *et al.* conducted studies also *in vivo* with A431 tumor xenografts in athymic mice and they found that this antibody is capable of eradicating established tumors in a single-cycle treatment. Thus, at high dose, it reduced EGFR phosphorylation and at low dose, ADCC has a significant role also *in vivo* (71).

Lammerts Van Bueren *et al.* investigated *in vivo* zalutumumab clearance in cynomolgus monkeys; they observed nonlinear clearance accelerated at low doses but not at high doses. To understand this mechanism, they performed *in vitro* experiments with EGFR-overexpressing A431 cells and they found that *in vitro* binding of zalutumumab to EGFR resulted in internalization with the

antibody at the cell membrane and in cytoplasmic microvesicles, and in antibody catabolism by EGFR-overexpressing cells. This binding, internalization, and degradation can result in nonlinear clearance of zalutumumab, as observed in monkeys (75).

8. PANITUMUMAB

8.1 .Mechanism of action

Panitumumab, previously known as ABX-EGF (clone E7.6.3), is a totally human IgG2 monoclonal antibody anti – EGFR (63, 76). This antibody has been developed using XenoMouse (Abgenix, Fremont, CA, USA) technology, where the human immunoglobulin genes have been introduced into mice engineered to lack functional mouse immunoglobulin genes (76, 77).

Panitumumab (Vectibix) was approved for monotherapy of relapsed ⁄ refractory mCRC by the US Food and Drug Administration (FDA) in September 2006 and conditionally approved (in patients with tumours harbouring wild-type KRAS) by the European Medicines Agency (EMEA) in December 2007 (77).

This antibody binds to surface exposed amino acids in the extracellular domain of the EGFR (domain III) [AACR-NCI-EORTC International Conference: Molecular Targets and Cancer Therapeutics, Oct 22-26, 2007, unpublished data]. This binding happens with high affinity (Kd $5 \cdot 10^{-11}$ mol⁄L), and therefore can inhibit or dissociate the binding of EGF and TGF-α to the receptor very effectively and can deprive cells from receiving essential growth and survival stimuli. Yang *et al.* investigated the effect of panitumumab on the *in vitro* proliferation of the EGFR-expressing tumor cell line A431 and they found that this antibody abolishes the signaling events triggered by EGF or TGF-alpha , included EGFR autophosphorylation, increases extracellular acidification rate, and inhibits cell proliferation (78). Others studies showed that the complex receptor–antibody is internalizated (77). The panitumumab does not act as an agonist and does not activate cells on binding to the receptor (78).

8.2. Biological properties

López-Albaitero *et al*. analyzed the effect of panitumumab in PCI-15B, squamous cell carcinoma of the head and neck (SCCHN) cell line, EGFR-overexpressing. They utilized this antibody at concentrations of 10 microg/mL and the results indicate that it does not induce tumor cell apoptosis or cytolysis (79).

Further studies have been conducted to analyse the effect of panitumumab on tumor cell growth *in vivo*. Yang *et al.* evaluated its ability to prevent the formation of A431 tumor xenografts in athymic mice and they found that this treatment did not only arrest the growth of the tumors but also caused continuous tumor regression resulting in a complete tumor eradication in all of the mice treated, without the need for concomitant chemotherapy (78). Yang *et al.* have extended their studies in other cell lines characterized by different levels of EGFR expression; in fact they inoculated subcutaneously into nude mice tumor cells derived from kidney (SK-RC-29), pancreas (BxPC-3, HS766T, and HPAC), prostate (PC3), ovary (IGROVI) and colon (HT-29 and SW707) and they observed that panitumumab inhibits growth of tumors expressing 17000 or more EGFRs per cell (76).

The mechanism, responsible for these cytocidal effects of panitumumab *in vivo,* is not yet elucidated and may involve the downregulation of EGFR expression, caused by receptor internalization, induction of apoptosis triggered by blocking EGFR signaling pathways and induction of cell cycle arrest. Moreover, this antibody has antiangiogenesis effects, due to inhibition of angiogenic growth factors production , as vascular endothelial growth factor and interleukin-8, and it has anti-invasive and antimetastatic effects caused by inhibition of matrix metalloproteinase production (80). These mechanisms are similar to those shown to be triggered by others antibodies in cultured cells (78).

The panitumumab is a human IgG2 antibody and lacks effector functions, complement-dependent and antibody-dependent cell-mediated cytotoxicities (ADCC), but López-Albaitero *et al.* showed that this antibody, as cetuximab, mediate immune activation through ADCC in SCCHN cell lines in the presence of peripheral blood lymphocytes, at the dose similar to those required for complete pEGFR blockade (76, 79, 80). Moreover, recent studies conducted by Schneider-Merck *et al.* found that panitumumab was as effective as zalutumumab (a human IgG1) in recruiting ADCC by cells of myeloid lineage (i.e., neutrophils and monocytes) in contrast to NK cell-mediated ADCC, which was only induced by the IgG1 Ab (81).

9. PERSPECTIVES

In this review currently known pre-clinical data on the antitumor effects of recombinant, human/mouse chimeric monoclonal antibodies (MoAbs) against EGFR are summarized. Both antibodies in clinical practice and some of those in phase I/III clinical trials are described however, this analysis together with the well known not exciting response of cancer patients, clinical responses have been observed in only 15% of patients treated, evidenced that a greater effort should be made to search more reliable predictive factors for patients selection and to develop new MoAbs with increased cytotoxic actions.

In confirmation of our idea is the importance of known predictors as K-Ras which significantly increased the capacity to select patients with advanced colorectal cancer to be treated with cetuximab (40).

For what concerns the future in the chemistry of monoclonal antibodies, it is linked to obtaining a more stable and homogeneous structures which have increased properties. Currently, the strategies followed are to produce antibodies: i) with improved pharmaceutical properties, through the removal by mutation of instability or aggregation hot spots in the antibody complementarity-determining regions and the use of hinge-stabilized or aglycosylated IgG4, ii) with improved antibody functions,

such as increased binding affinity of antibody Fv to the target, altered glycosylation status, increased modulation of ADCC, increased serum half-life and so on, iii) of second and third generation, by conjugating them to other drugs mainly immunological and cytotoxic ones and iv) directed against two different tumour associated or immunological antigen targets (bispecific antibodies) (82). Finally, another strategy is to obtain recombinant polyclonal or oligoclonal antibodies directed against the same or different targets; an example is Sym004 (Symphogen A/S), which is a controlled mixture of two EGFR-specific antibodies with an higher response as respect to cetuximab and panitumumab alone (82). This compound is in Phase I clinical trial for treatment of patients with EGFR+ breast cancer (83).

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