

Bivalent binding by intermediate affinity of nimotuzumab

A contribution to explain antibody clinical profile

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Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; mAb, monoclonal antibody; FBS, fetal bovine serum; FITC, fluorescein iso-thiocyanate; HRCE, human renal cortical epithelial cells; HEC, human epidermal cell suspensions; PI, propidium iodide; K_D , dissociation constant

Nimotuzumab is an EGFR-targeting antibody that has demonstrated encouraging clinical results in the absence of severe side-effects observed with other approved anti-EGFR antibodies. We investigated whether different clinical behavior of nimotuzumab is related to its bivalent/monovalent binding profile. Binding properties of nimotuzumab and cetuximab, the most development of anti-EGFR antibodies, were studied in vitro using chip surfaces and cells with varying EGFR expression levels. Experimental observations demonstrated that in contrast to cetuximab, the intrinsic properties of nimotuzumab required bivalent binding for stable attachment to the cellular surface, leading to nimotuzumab selectively binding to cells that express moderate to high EGFR expression levels. At these conditions, both antibodies bonded bivalently and accumulated to similar degrees. When EGFR density is low, nimotuzumab monovalent interaction was transient, whereas cetuximab continued to interact strongly with the receptors. We compared the in vitro antitumor efficacy of nimotuzumab and cetuximab. Cetuximab decreased the cell viability and induced apoptosis for all the tested cell lines, effects which did not depend on EGFR expression level. In contrast, nimotuzumab also provoked significant anti-cellular effects, but its antitumor capacity decreased together with EGFR expression level. Cetuximab Fab fragment was able to impact tumor cell survival, whereas nimotuzumab fragment totally lost this effect. Tumor-xenograft experiments using cells with a high EGFR expression revealed similar tumor growth inhibiting effects for both antibodies. This study suggests an explanation for nimotuzumab clinical profile, whereby antitumor activity is obtained in absence of severe toxicities due to its properties of bivalent binding to EGFR.

Introduction

Targeted therapies constitute a major trend in new drug development for cancer treatment, fueled by the increasing knowledge of the genetic alterations leading to malignant transformation and progression. Among the molecular targets that are currently in clinical evaluation, the epidermal growth factor receptor (EGFR), also known as HER1, has been widely validated and several EGFR antagonists, including oral small molecule tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (mAbs) have been approved by the FDA.¹ Five anti-EGFR mAbs have been evaluated in the phase II or phase III clinical studies with positive or encouraging results. Among them, cetuximab (a chimeric IgG1 previously known as C225), has been extensively studied in

a variety of tumor types.² Moreover, panitumumab, matuzumab and zalutumumab have been tested in several different types of EGFR-positive tumors.³ Nimotuzumab (a humanized IgG1 previously known as h-R3) has been investigated in patients with head and neck^{4,5} and glioma tumors,⁶⁻⁸ and several clinical trials for other tumor indications are ongoing.⁹⁻¹²

EGFR is commonly overexpressed in human epithelial tumors as compared with the expression levels observed in normal tissues such as skin and kidney. A drawback of EGFR-targeted therapies is the severe skin rash toxicity and other related adverse events that they provoke in renal cells and gastro-intestinal mucosa. The side-effects are believed to be caused by reacting of the anti-EGFR targeting drugs to the receptor in tissues other than the tumor.^{13,14} It has been postulated that there is a direct correlation

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between the clinical efficacy of EGFR-targeting agents and the severity of the induced acneiform skin rash.^{15,16} In clinical trials completed to date, nimotuzumab has demonstrated a unique safety clinical profile, where antitumor activity was observed with a very low toxicity profile.⁴⁻⁶ Notably, the grade 3 and 4 acneiform rash commonly associated with other anti-EGFR antibodies is absent.¹⁷

We have previously formulated a mathematical model based on phase II clinical trial data, to explain why nimotuzumab may have good clinical efficacy without showing severe adverse events.⁴ According to this theoretical model, antibodies with intermediate affinities would have a higher ratio of accumulation in tumors (which have higher EGFR expression levels) with respect to normal tissues, as compared to high affinity antibodies. Nimotuzumab, whose monovalent Fab fragment has a tenfold lower affinity for the extracellular domain of EGFR than the Fab of cetuximab,¹⁸ would be within this favorable “affinity window.”

Emerging evidence suggests that the ability of antibodies to bind bivalently (i.e., binding with both antibody arms to two targets simultaneously) is essential for maintaining prolonged drug residence in tumors and is an important feature for inhibiting tumor cell proliferation.¹⁹⁻²¹ Formation of bivalent bonds is dependent on target density and antibody binding kinetics, such that increased receptor density on the cellular surface facilitates formation of bivalent bonds. In addition, there is no clinical evidence from studies with anti-EGFR antibodies that higher affinity (i.e., strength of monovalent interaction) leads to greater efficacy though stronger monovalent binding could lead to higher toxicities. When patients carrying advanced squamous cell carcinoma head and neck (SCCHN) were treated with nimotuzumab in combination with radiotherapy, almost all adverse events were graded as I or II. Irradiation toxicity was not augmented with nimotuzumab and, no skin rash was found.⁵ However, treatment of similar patient population with cetuximab and radiation provoked that 87% of patients developed acneiform rash.²² Even more, a recent EORTC survey indicates that grades 3 and 4 dermatitis occur in approximately 49% of SCCHN patients during radiation therapy with concurrent cetuximab.²³ Thus, the affinity and avidity of the binding interactions may play critical roles in the clinical profile of anti-EGFR antibodies.

In this paper, binding properties of nimotuzumab, in particular bivalent and monovalent binding to tumor cells having differential EGFR expression, and the effect on such tumor cells, in comparison with cetuximab were investigated. Binding patterns of nimotuzumab and cetuximab to cells from healthy tissues (renal and skin) were also examined.

Results

Nimotuzumab and cetuximab display differences in monovalent/bivalent binding profiles. The capability of nimotuzumab and cetuximab, as well as of their Fab fragments, to bind to tumor cells having different expression levels of the EGFR was first analyzed by flow cytometry using five different human tumor cell lines (A431, H125, U1810, MDA-MB-231 and U1906). EGFR

expression levels in these cells were measured by western blot and flow cytometry analysis. As shown in **Figure 1A**, the EGFR expression decreased from the A431 to the MDA-MB-231 cell lines, whereas its expression was not detected in U1906 cells. Similar results were obtained when surface EGFR expression was analyzed (**Fig. 1B**). These results are in agreement with previous reports showing that the A431 cells express 2–3 × 10⁶ EGFR molecules per cell;³² the H125 and U1810 cells express 2.1 × 10⁵ and 7 × 10³ EGFR molecules per cell, respectively,³³ whereas the MDA-MB-231 cells have a weak expression,³⁴ and the U1906 cell line does not express the EGFR at all.³³

The different cell lines were incubated with increasing concentrations of nimotuzumab, cetuximab, or their Fabs. The ratios between the nimotuzumab and cetuximab binding capabilities were measured by flow cytometry. Marked differences in antibody binding patterns were observed as a function of EGFR density and binding mode (bivalent vs. monovalent). As shown in **Figure 1C**, cetuximab demonstrated consistent binding to all cell lines independent of EGFR expression. In contrast, the slope fluorescence mean intensity (FMI) curve indicating nimotuzumab accumulation was significantly and positively affected by increasing EGFR expression. Nimotuzumab and cetuximab binding curves were remarkably similar at all concentration levels for cells lines with moderate to high receptor expression (H125 and A431). The binding experiments carried out with the monovalent Fab fragments revealed that the cetuximab Fab binding is independent of EGFR number. In contrast, monovalent binding is not efficient with nimotuzumab Fab, with minimal level of binding at the various EGFR expression levels and fragment concentrations (**Fig. 1D**).

To study the interaction between antibodies and EGFR collecting data continuously in real time without the need for enzymatic modification of the antibodies, we conducted a study with Surface Plasmon Resonance. The binding of nimotuzumab and cetuximab was compared under conditions only allowing monovalent (i.e., low EGFR density) or bivalent (i.e., high EGFR density) binding. Consistent with flow cytometry results, under monovalent conditions (**Fig. 2A**), significant differences were observed between the levels of binding, with nimotuzumab demonstrating the lowest response. Under conditions representing high EGFR density by allowing bivalent interactions (**Fig. 2B**), the anti-EGFR antibodies behaved remarkably similar, accumulating to equivalent levels.

Nimotuzumab binds minimally to renal and skin non-malignant cells. We tested also the recognition of normal tissues by nimotuzumab, cetuximab and their Fab fragments by flow cytometry. EGFR expression of human renal cortical epithelial cells (HRCE) and human epidermal cell suspension (HEC) was characterized by protein immunoblot using A431 cells as positive control. As expected from the results previously reported by Real et al.³⁵ HRCE and HEC had similar EGFR levels and their EGFR expression was ≈100-fold lower than A431 cells (**Fig. 3A and B**). Binding of cetuximab to HRCE and HEC was significant and concentration dependent. Nimotuzumab was able to recognize primary cultures of normal renal and skin cells but its recognition capacity was lower by a factor of 2–3 compared to cetuximab (**Fig. 3C**). Cetuximab Fab bound to these cells and

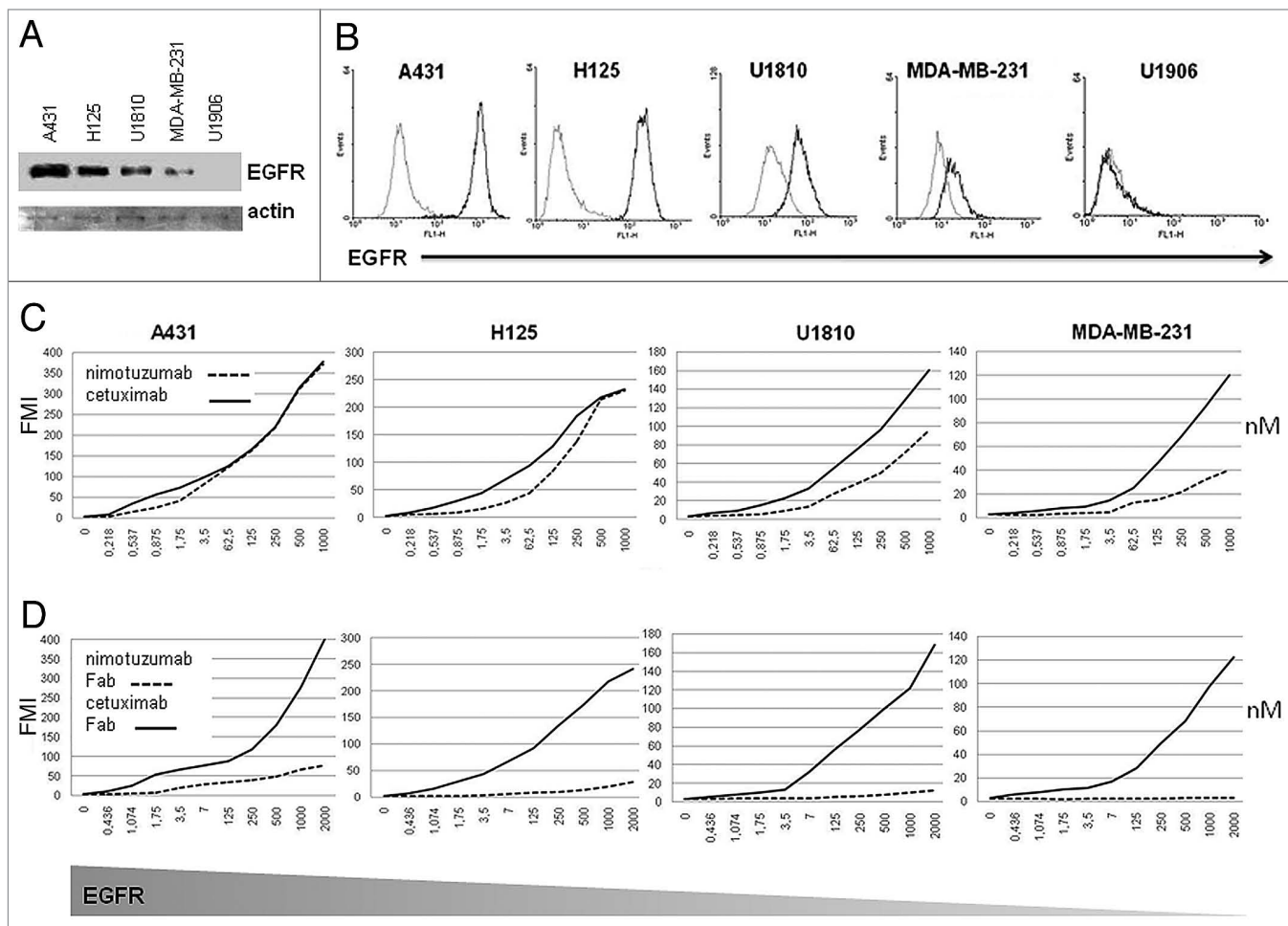


Figure 1. Binding of nimotuzumab, cetuximab and their monovalent fragments to human tumor cell lines with different EGFR expression. (A) Immunoblot for the total EGFR and actin were performed for the indicated tumor cells. (B) Tumor cells (grey histograms) were stained with an anti-EGFR antibody (black histograms), followed by incubation with FITC-conjugated rabbit anti-rat IgG-H&L. (C) Tumor cell lines were incubated with nimotuzumab or cetuximab antibodies followed by FITC-conjugated anti-human IgG (Fab specific). (D) Tumor cell lines were labeled with two-fold higher concentration of nimotuzumab or cetuximab Fabs than the concentration used for whole antibodies followed by FITC-conjugated anti-human IgG (Fab specific). (C and D) For each point represents the mean of triplicate wells. The data were expressed as fluorescence mean intensity (FMI); and the U1906 cell line was used as negative control for EGFR expression (data not shown). All experiments were performed at least three times with similar results. Representative data are shown.

binding was similar to that observed with the whole mAb (equimolar concentrations). Nimotuzumab Fab binding to HRCE and HEC was undetectable regardless of the Fab concentrations (Fig. 3D).

Nimotuzumab's anticellular effect depends on EGFR density. To further obtain insights on the impact of the differences in monovalent and bivalent binding between nimotuzumab and cetuximab, we first tested their effect on cell viability by MTT assays for the A431, H125, U1810 and MDA-MB-231 cells. As shown in Figure 4A, cetuximab decreased the cell viability for all the tested cell lines in a concentration-dependent manner. The percentage of viable cells recovered after treatment with the highest concentration of cetuximab was similar for the four cell types, i.e., its effect did not depend on the EGFR expression level. In contrast, the capacity of nimotuzumab to reduce the viability became weaker as the EGFR expression level decreased. Next,

we investigated the capability of nimotuzumab and cetuximab to induce apoptosis in the tumor cell lines included in this study. For this purpose, we measured the level of DNA fragmentation by propidium iodide (PI) staining. Treatment with cetuximab at 70 nM provoked an increase in the number of A431, H125, U1810 and MDA-MB-231 apoptotic cells, as compared with non-treated cells (Fig. 4B). The values of the percentage of cells with DNA fragmentation were similar for all the cell lines. Treatment with nimotuzumab also produced an increase of the percentage of apoptotic cells in the A431, H125, U1810 and MDA-MB-231 lines, but its effect decreased in parallel with the EGFR expression levels (Fig. 4B).

In addition, we compared the in vitro antitumor efficacy of the antibodies monovalent fragments on A431 cells. As shown in Figure 4A and B, in contrast to cetuximab Fab, nimotuzumab monovalent fragment totally lost its capacity to decrease cell

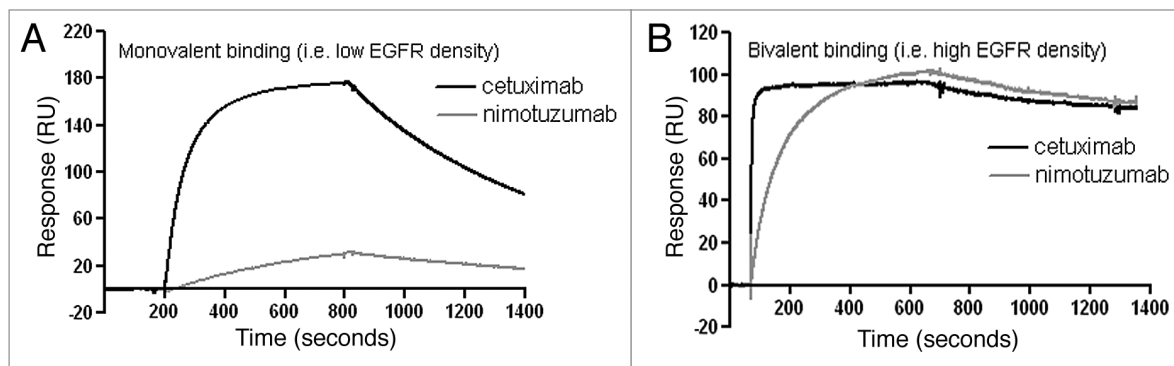


Figure 2. Examination of cetuximab and nimotuzumab binding kinetics using surface plasmon resonance. (A) Under monovalent binding conditions (intended to represent binding to cells with low EGFR expression) antibodies demonstrated significant variance in the level of accumulation, with very little binding occurring with nimotuzumab; ligand-anti-EGFR antibodies, analyte-100 nM EGFR monomer. (B) Under bivalent binding conditions (intended to represent binding to cells with high EGFR expression), antibodies behaved similarly and accumulated to the equivalent levels; ligand-Fc-EGFR chimera (dimer); analyte-100 nM anti-EGFR antibodies. Experiments were performed three times with similar results. Representative data are shown.

viability or induce apoptosis. In concordance with the results reported by other groups,^{21,36} cetuximab Fab displayed weaker anticellular effects against A431 cells than cetuximab.

Finally, we examined the effects of nimotuzumab and cetuximab on cells that overexpress EGFR *in vivo* using the A431 xenograft model. A comparable antitumor effect was noted in the groups treated with nimotuzumab and cetuximab (Fig. 5). Conversely, in the control group, the tumors continued growing and the tumor volume at the end of the experiment was almost twice the size of the implants at the beginning of treatment. Similar *in vivo* results had been previously reported from comparative studies using both anti-EGFR antibodies.³⁷

Discussion

Nimotuzumab is one of the very few anti-EGFR antibodies that have been approved for therapeutic use in cancer treatment. It recognizes domain III of the extracellular region of EGFR, within an area that overlaps with both the surface patch recognized by cetuximab and the binding site for EGF.¹⁸ Encouraging activity of nimotuzumab has been demonstrated in *in vivo* models³⁸ and multiple clinical trials, mainly phase I and phase II trials that have focused on head and neck and brain malignancies.⁴⁻⁷ In total, nimotuzumab has been administered to more than 4,000 patients and notably no evidence of severe skin rash has been reported.³⁹ Even though nimotuzumab produces an inhibition of the EGFR phosphorylation in normal skin cells, no significant changes in activation status of downstream molecules were found. Besides, the characteristic lymphocytic infiltrates, folliculitis or perifolliculitis induced by other anti-EGFR antibodies have not been observed in nimotuzumab-treated skin patient samples.^{40,41} In this regard, it has been reported that inflammatory response is responsible for many of the signs and symptoms that are associated with skin toxicity.^{42,43} However, the degree of EGFR inhibition enough to cause inflammatory reaction that leads to rash is yet unknown. Several studies using skin samples from patients treated with anti-EGFR agents revealed

that the reduction of EGFR phosphorylation was unrelated to toxicity.^{44,45}

A current paradigm in targeted antitumor antibody therapy is to pursue the highest affinity drug candidates. While this may hold for antibodies targeting tumor-specific antigens, for tumor-associated antigens like EGFR, *i.e.*, molecules that are overexpressed in tumors but are also ubiquitously expressed in normal tissues, this paradigm may not be valid as biodistribution and tumor penetration properties may be adversely affected very high affinity.⁴⁶

A mathematical model was previously constructed that predicted an “affinity window” (K_D between 10^{-7} and 10^{-9} M) for anti-EGFR antibodies for optimal therapeutic index.⁴ Affinity of nimotuzumab is ten times lower than the affinity of cetuximab (K_D for the Fab fragments: 2.1×10^{-8} and 2.3×10^{-9} M, respectively)^{18,47} and would fall within the predicted optimal affinity window.

This study provides experimental results that extend the “affinity window” hypothesis by adding avidity component to the model. Our results suggest that antibodies with intermediate affinity would preferentially target tissues overexpressing target antigen because these antibodies rely on bivalent binding for stable attachment to cellular surface. Zuckier et al.⁴⁸ investigated the influence of the affinity and antigen density on antibody localization. In that study only high affinity Fab fragments bound effectively, independently of the antigen density. In contrast, low affinity antibodies required the avidity conferred by bivalent binding for effective attachment, which can occur only if antigen density is elevated.

We report similar results when comparing binding of nimotuzumab, cetuximab and their corresponding monovalent fragments to a variety of human tumor with different levels of EGFR expression. In our studies, cetuximab Fab fragment bound to all cell lines irrespective of the EGFR expression level, while the nimotuzumab Fab possessed a significantly diminished binding to cells with high EGFR content ($\approx 10^6$ molecules/cell). When the binding capacity of the intact antibodies was tested, cetuximab

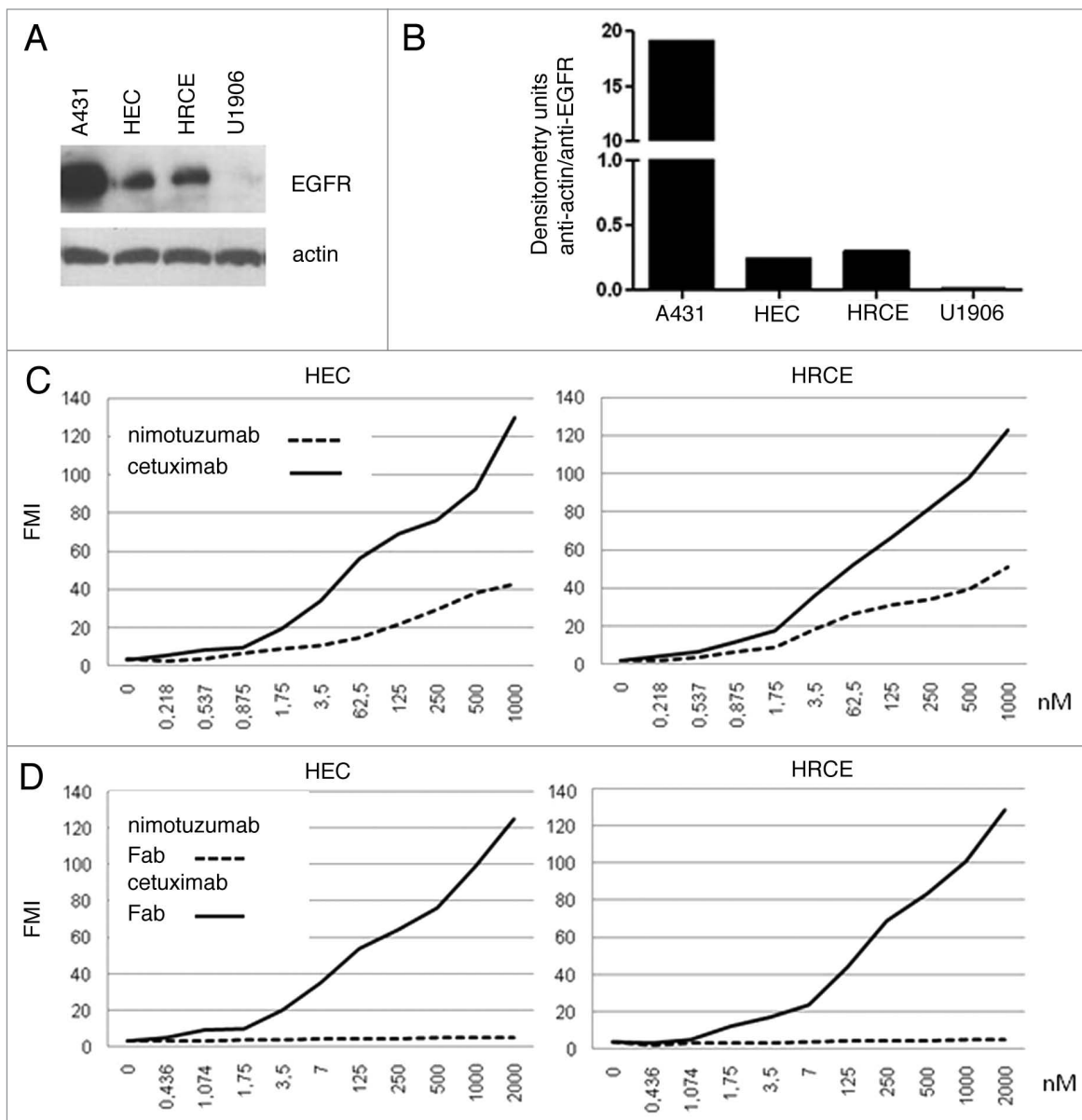


Figure 3. Binding of nimotuzumab, cetuximab and their monovalent fragments to renal and skin normal cells. (A) Immunoblots for the total EGFR and actin were performed for the indicated cells. U1906 was used as negative control for EGFR expression. (B) Graphs represent the relationship between the densitometry units obtained with the anti-EGFR antibody and the same blot reprobed with anti-actin antibody. (C) HRCE and HEC were incubated with nimotuzumab or cetuximab antibodies followed by FITC-conjugated anti-human IgG (Fab specific). (D) HRCE and HEC were labeled with two-fold higher concentration of nimotuzumab or cetuximab Fabs than the concentration used for whole antibodies followed by FITC-conjugated anti-human IgG (Fab specific). For (C and D) each point represents the mean of triplicate wells. The data were expressed as fluorescence mean intensity (FMI). All experiments were performed at least three times with similar results. One representative experiment out of three is shown.

demonstrated consistent binding to all cell lines, independent of the relative level of EGFR expression. In contrast, the slope FMI curve indicating nimotuzumab accumulation was significantly and positively affected by increasing EGFR expression, such that at medium to high EGFR expression, cetuximab and nimotuzumab binding curves had similar slopes and close to each other. In addition, cetuximab can achieve both monovalent and bivalent binding to renal and skin normal cells. However, we could only detect a minimal recognition and reactivity of non-malignant cells with

nimotuzumab. These marked differences in binding patterns of nimotuzumab and cetuximab to cells from normal tissues may contribute to explain their differential toxicity profiles. However, further *in vitro* experiments should be designed to determine nimotuzumab effect on normal cells. Previous reports have demonstrated that cetuximab efficiently inhibited the *in vitro* EGFR activation in human epidermal keratinocytes cultures.⁴⁹

From these results, we could predict that nimotuzumab will have preferential uptake in EGFR overexpressing tumors,

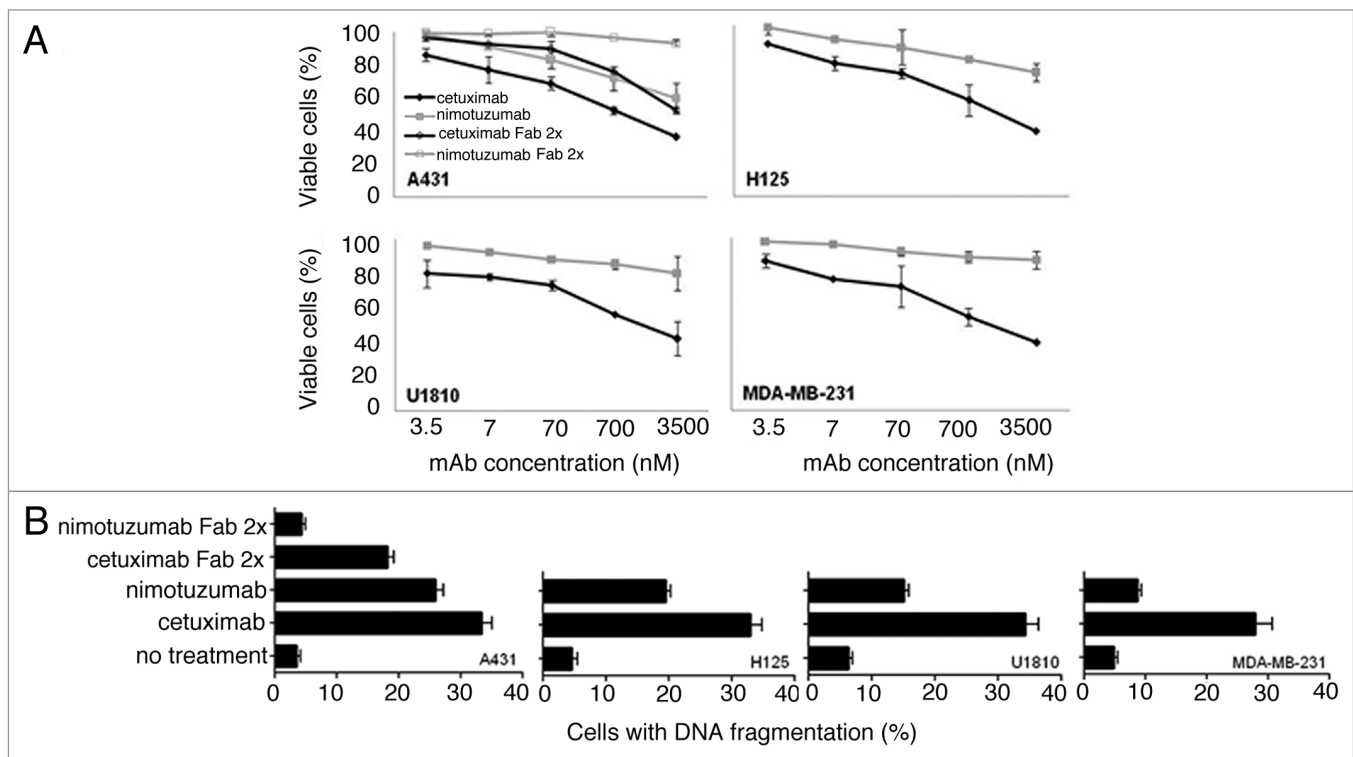


Figure 4. In vitro effects of nimotuzumab and cetuximab on cells with different EGFR expression levels. (A) Cells were treated with antibodies (3.5–3,500 nM) or their Fab fragments (7–7,000 nM) for 96 h. Cell viability was measured by MTT assay. Non-treated cells were included as a maximum cell viability point. The percentage of viable cells was determined using the following formula: $[\text{treated cells Abs}_{540-620 \text{ nm}} / \text{non-treated cells Abs}_{540-620 \text{ nm}}] \times 100$. Each point represents the mean of triplicate wells. The error bars indicate the SD. (B) Cells treated with antibodies (70 nM) or their monovalent Fab fragments (140 nM) for 24 h were fixed with ice-cold methanol/acetone and stained with PI (400 $\mu\text{g}/\text{ml}$). The percentage of cells with DNA fragmentation (apoptotic cells) was analyzed by flow cytometry. For both experiments the U1906 cell line was used as a negative control for EGFR expression and the fenretinide was used as positive control in the apoptosis assay (data not shown). One representative experiment out of three is shown in each case.

accompanied by low uptake in normal tissues (i.e., the skin and kidney) and consequently reduced incidence of toxicities. Present findings also suggest a potential synergy of nimotuzumab with agents that increase EGFR expression, such as radiation containing regimens. Recent reports give support to this hypothesis. Akashi and coworkers showed that binding of nimotuzumab and subsequent inhibition of the EGFR phosphorylation are detected only for tumor cells lines with medium or high levels of EGFR expression (10^4 receptors per cell or higher).⁵⁰ On the other hand, Diaz and collaborators demonstrated the superiority of combined treatment of nimotuzumab and radiation over each single therapy against glioblastoma multiforme using a murine in vivo model.⁵¹ In a recent report of a multicenter, double blind, randomized clinical trial, a significant survival improvement was observed for patients with EGFR-positive tumors that were treated with nimotuzumab.⁵ It remains to be shown whether the EGFR expression level is a predictive marker of nimotuzumab's clinical efficacy, in contrast to high affinity antibodies like cetuximab, for which it has been shown that the EGFR expression level is not a predictive marker of clinical benefit.⁵²

It has also been hypothesized that very high affinity interactions between antibodies and tumor antigens may impair efficient intra-tumor penetration, thus impairing in vivo targeting.⁵³

Adams et al.⁵⁴ reported that intrinsic affinity properties regulate the quantitative delivery of antitumor single-chain Fv (scFv) molecules from the vasculature into tumor masses. While lower affinity molecules exhibited efficient tumor staining, higher affinity scFv molecules were primarily retained in the tumor perivascular regions. In addition, in vitro experiments have showed that bivalent binding enhances inhibition of tumor cell proliferation,^{36,55} EGFR downregulation²¹ and EGFR signaling inhibition.²⁰ Our results confirm these reports; monovalent Fab fragment of cetuximab had weaker anticellular effect than cetuximab on A431 cells. Thus, bivalent binding by intermediate affinity anti-EGFR antibodies provides a pharmacological advantage.

A prior study also demonstrated a dose-dependent increase in circulating TGF α as a result of the autocrine loop disruption by cetuximab treatment.⁵⁶ Comparing effects of cetuximab and nimotuzumab on ligand release, the same group found a decrease in cell medium accumulated TGF α when MDA-MB-231 cells were incubated with the nimotuzumab compared to cetuximab (Tony Mutsaers, personal communication). Differences in binding profiles of both antibodies to MDA-MB-231, a low EGFR-expressing tumor cell line, helps explain the differences in preventing TGF α internalization. These findings are especially relevant due to inhibition of TGF α local capture by EGFR in the

dermis allowing the ligand to diffuse and act as chemotactic factor, as suggested by Coffey et al.⁵⁷ This phenomenon may account for the acneiform eruptions observed with cetuximab treatment where inflammatory cells have been observed.

Based on the crystal structures of the nimotuzumab Fab fragment and the extracellular region of EGFR⁵⁸ a computational model of the complex between the two molecules was constructed.¹⁸ According to this model, nimotuzumab inhibits the EGF-dependent EGFR activation, but may not alter the basal equilibrium between the “tethered” (inactive) and the active (ready for ligand binding and dimerization) receptor conformations. There are several reports that demonstrate the existence of ligand-independent EGFR activation in normal tissues,⁵⁹ which is needed for the survival of normal epithelial cells.⁶⁰ This mechanism of inhibiting the EGFR signaling that allows basal activity of EGFR by nimotuzumab could be another factor contributing to the drug’s benign toxicity profile. Recent clinical findings from Rojo and collaborators, mentioned above, could indicate the validity of this model.⁴⁰ Nevertheless, experiments to establish the precise binding confirmation of nimotuzumab bound to EGFR are being conducted.

In Figure 6 are summarized experimental observations discussed above in a schematic representation. When EGFR expression is moderate to high, all antibodies behave similarly in that they bind with both arms and accumulate to similar degrees. When EGFR density is low, such as on normal tissues, cetuximab continue to interact strongly with the receptors. In contrast, nimotuzumab monovalent interaction is transient. This model would explain the preferential uptake of nimotuzumab in EGFR overexpressing tumors and its low toxicity profile, providing a mechanistic basis for the previously postulated “affinity window” hypothesis.⁴

In summary, we have provided elements to understand the dynamic interaction of nimotuzumab with the receptor in normal and tumor cells. Our findings suggest that the intrinsic properties of nimotuzumab require bivalent binding for stable attachment, which leads to nimotuzumab selectively binding to EGFR overexpressing cells. This EGFR-density dependence allows us to explain some clinical observations related to the pharmacological behavior of nimotuzumab, specifically, antitumor activity without severe skin and renal toxicities. We propose bivalent binding model as a refinement of the previous “window affinity” hypothesis. This type of translational research might have clinical impact providing the rationale for antibody chronic administration and therapeutic combinations with a low toxicity profile. Specifically, toxicity limits the ability to co-administer currently approved anti-EGFR antibodies with conventional therapies and other targeted agents (e.g., EGFR TKIs, anti-VEGF antagonists).

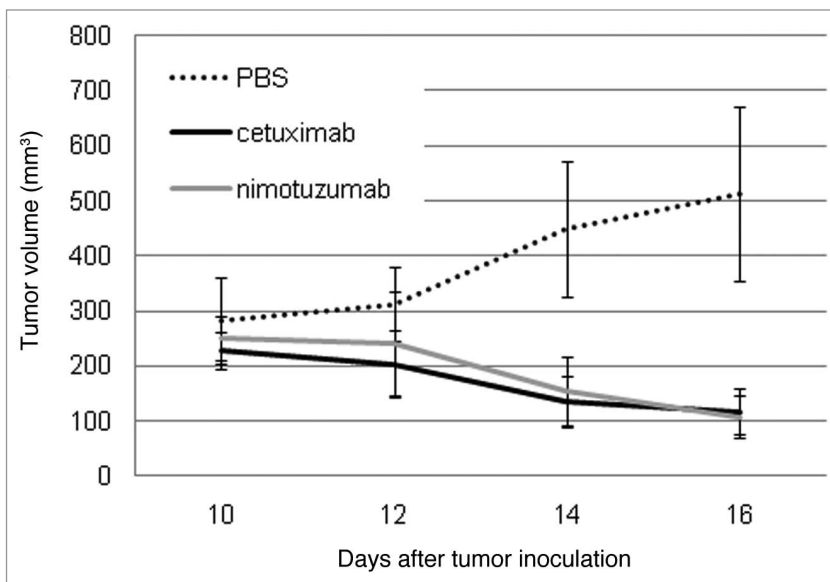


Figure 5. Effect of anti-EGFR antibodies on the growth of established A431 xenografts. Tumor cells were inoculated subcutaneously in SCID mice. Antibody treatment (1 mg/inoculation) consisted of four intraperitoneal injections every 48 h, beginning on day 10 after tumor inoculation. The control group received 1x PBS. Tumor size was measured every injection day and the volume was determined using the following formula: $\frac{1}{2}$ larger diameter x (smaller diameter).² Each point represents the mean of four mice. The error bars indicate the SD. One representative experiment of two is shown.

Nimotuzumab would be better suited for such combination treatments and combining nimotuzumab with TKIs is a particularly appealing strategy. On the other hand, this study addressed us to evaluate EGFR expression in nimotuzumab-treated patients and, to define its role as a possible predictive marker.

Materials and Methods

Antibodies and reagents. The humanized anti-EGFR mAb nimotuzumab (h-R3)²⁴ was obtained at the Center of Molecular Immunology. The anti-EGFR mAb cetuximab (C225), a human-mouse chimeric form of the original mouse monoclonal 225, initially described by Kawamoto et al.²⁵ was manufactured by Merck Pharma GmbH. Antibodies to total EGFR and actin were used for western blot experiments and obtained from Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology). Antibodies anti-human IgG (Fab specific) conjugated to FITC (fluorescein iso-thiocyanate) and anti- extracellular domain of human EGFR were used for flow cytometry analysis and purchased from Sigma and Abcam, respectively. The synthetic retinoid fenretinide was provided by Calbiochem. Human EGF was purchased from Center for Genetic Engineering and Biotechnology.

Purification of Fab fragments. The nimotuzumab and cetuximab Fab fragments were generated by papain digestion. The mAbs (2 mg/ml) were incubated with papain (1:100 w:w, Sigma) at 37°C for 4 h and the reaction was stopped with 20 mM iodoacetamide (Sigma). Undigested mAb and Fc fragments were removed by passing the mixture by a protein-A column. The Fab fraction was then dialyzed extensively against 1x

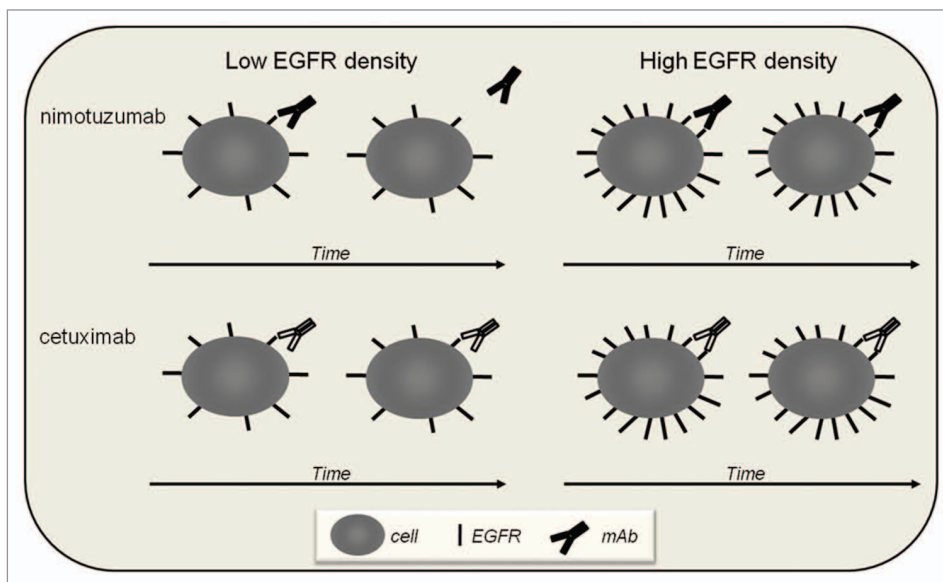


Figure 6. Schematic representation of nimotuzumab binding properties as a function of EGFR density. Unlike cetuximab, nimotuzumab requires bivalent binding (two arms) for stable attachment, which leads to it selectively binding to cells expressing moderate to high EGFR levels. When EGFR density is low, such as in normal tissues, cetuximab continues to interact strongly with the receptors. In contrast, nimotuzumab monovalent interaction is transient, thus sparing healthy tissues and avoiding severe toxicities. When EGFR expression is moderate to high, all antibodies behave similarly in that they bind bivalently, which is the most stable mode of binding.

PBS, pH 7.4. The purity of Fabs was analyzed by 10% SDS-PAGE gels.

Cell lines and culture conditions. The following cell lines were used on the experiments: the A431 human epidermoid carcinoma (CRL-1555, ATCC), the MDA-MB-231 human breast adenocarcinoma (HTB-26, ATCC), the U1906 human small cell lung cancer,²⁶ the H125 human lung adenocarcinoma and the U1810 human non-small cell lung cancer.²⁷ Cell lines were cultured in DMEM:F12 (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Life Technologies). Human renal cortical epithelial cells (HRCE) were obtained from Lonza Group and maintained in growth basal medium according to manufacturer's instructions.

Preparation of human epidermal cell suspensions. Human epidermal cell suspensions (HEC) were prepared from normal skin (plastic surgery of breast). To facilitate the removal of epidermis from dermis, skin biopsies were first placed in 0.5% cold acetic acid for 48 h. Cell suspensions were obtained from epidermis disc by trypsinization (0.3% trypsin at 37°C for 1 h), according to the method described previously.²⁸ Viability, as determined by trypan blue exclusion, was >85%.

Western blot analysis. Cell lysates were prepared in RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with 50 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA and 1 mM phenylmethylsulphonylfluoride that were freshly added to the lysis solution before each experiment. Protein concentrations were determined according to bicinchoninic acid protein assay kit (Pierce). Cell extracts were applied to 7.5% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes

(Gelman). Polyvinylidene difluoride membranes were blocked with NEG-T buffer (0.15 M NaCl, 5 mM EDTA, 500 mM Tris-HCl [pH 7.5], 0.02% Tween 20, 0.04% gelatin) and incubated with the primary antibodies described above. The protein content was visualized using horseradish peroxidase-conjugated secondary antibodies (BD Biosciences) followed by Chemiluminescent Substrate (Pierce).

Flow cytometry analysis for EGFR recognition. EGFR surface expression on human tumor cell lines was analyzed by flow cytometry using the specific antibody described above. Also, cells were incubated with nimotuzumab/cetuximab mAbs (0.218–1,000 nM) or with two-fold higher concentration of nimotuzumab/cetuximab Fabs than the concentration used for whole antibodies followed by FITC-conjugated anti-human IgG (Fab specific, 1:200 dilution). Data

were obtained with a FACScan Flow cytometer (BD Biosciences) by collecting a minimum of 10,000 events and analyzed using the WinMDI software. The data were expressed as fluorescence mean intensity (FMI).

Surface plasmon resonance. Antibody binding kinetics under monovalent (representing low EGFR expression) and bivalent (representing high EGFR expression) binding conditions were examined using Surface Plasmon Resonance (SPR; Biacore 3000). To examine bivalent binding conditions, CM5 chip surface was coupled with Fc-EGFR dimer using the standard primary amine coupling reaction wizard. Increasing concentrations of antibodies (nimotuzumab and cetuximab) were flowed for 10 min at 30 µl/min followed by 10 min dissociation phase. Monovalent binding properties of the antibodies were examined by coupling cetuximab, and nimotuzumab onto CM5 chip surface using standard primary amine coupling reaction wizard. Increasing concentrations of EGFR monomer were flowed for 10 min at 30 µl/min followed by 10 min dissociation phase. Normalization of this chip was performed as per Pär Säfsten et al.²⁹ Efficient regeneration of both chip surfaces was achieved using two consecutive injections of 10 µl of 50 mM NaOH. Kinetics confirmed by fitting with BiaEvaluation software.

Cell viability assay. A431, H125, U1810, MDA-MB-231 and U1906 cell viability was measured by MTT assay.³⁰ Tumor cells (1 × 10⁴) were seeded in DMEM:F12 10% FBS in flat-bottomed 96-well plates (Costar). After 12 h, the cells were switched to 1% FBS containing human EGF (500 pg/ml) and nimotuzumab/cetuximab mAbs (3.5–3,500 nM) or nimotuzumab/cetuximab Fabs (7–7,000 nM) were added; the cells were incubated for

48 h. The treatment was repeated for additional 48 h. Subsequently, MTT (1 mg/ml; Sigma) was added and incubated for 4 h at 37°C. Next, the formed formazan crystals were dissolved with DMSO. The absorbance difference 540–620 nm was determined using a Microwell System reader (Organon Teknika). Non-treated cells were included as a maximum cell viability point. The percentage of viable cells was determined using the following formula: $[\text{treated cells Abs}_{540-620 \text{ nm}} / \text{non-treated cells Abs}_{540-620 \text{ nm}}] \times 100$.

Apoptosis measurement. To measure the induction of apoptosis, DNA fragmentation was evaluated by propidium iodide (PI) staining.³¹ A431, H125, U1810, MDA-MB-231 and U1906 cells were plated (0.25×10^6) in DMEM:F12 10% FBS in 6-well plates (Costar). Twelve hours later, nimotuzumab/cetuximab mAbs (70 nM), nimotuzumab/cetuximab Fabs (140 nM) or fenretinide (15 μM) were added in DMEM:F12 1% FBS containing human EGF (500 pg/ml) and the cells were incubated for 48 h. The treatment was repeated for additional 48 h. Cells were fixed with ice-cold methanol/acetone (4:1) and stained by incubation with a solution containing 400 $\mu\text{g/ml}$ of PI (Sigma) and 100 $\mu\text{g/ml}$ RNase (Sigma). All analyses were performed on a FACScan Flow cytometer by collecting a minimum of 20,000 events and analyzed using the WinMDI 2.8 software.

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Tumor challenge assay. A431 cells ($5 \times 10^6/200 \mu\text{l}$ of 1x PBS) were inoculated subcutaneously (s.c.) into the right flank of 6–8 week old severe combined immunodeficient (SCID) mice (average weight, 23 g). After 10 days (tumor average size, 200 mm^3) the animals were randomly separated into three groups (four mice per group): untreated and nimotuzumab or cetuximab treated. Each treatment group consisted of four intraperitoneal (i.p) injections of 1x PBS (control group) or the indicated mAbs (1 mg/injection) every 48 hours. Treatment was stopped after a total of eight injections. Tumor size was measured every injection day and the volume was determined using the following formula: $\frac{1}{2}$ larger diameter x (smaller diameter).²

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Footnotes

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