

Binding properties of the anti-EGFR monoclonal antibody, nimotuzumab, limit its interaction with the EGFR in renal and epidermal cells

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SUMMARY

Severe skin rash and hypomagnesemia are hallmark toxicities of anti-EGFR antibodies cetuximab and panitumumab. Nimotuzumab is an EGFR-targeting Affinity-Optimized Antibody™ currently under investigation in advanced clinical trials. The antibody has demonstrated anti-tumor activity in the absence of severe skin, renal, GI mucosa and other toxicities commonly associated with other EGFR-targeting agents.

As a result of optimal affinity, attachment of nimotuzumab to the cellular surface has previously been reported to rely on bivalent binding, which is facilitated by EGFR overexpression.^[1] This intrinsic property was postulated to result in selective targeting of EGFR-overexpressing cancer cells and sparing of healthy tissues. In this study we examined EGFR expression of human renal cortical epithelial cells, human epidermal cells and A431 cancer cell line, and compared binding of nimotuzumab and cetuximab to these cells by Flowcytometric analysis (FACS). Human renal cortical epithelial and epidermal cells were found to express low levels of EGFR compared to A431 cancer cells. Nimotuzumab's binding to the epidermal and renal cells was several fold lower than that of cetuximab. Concurrently, binding of nimotuzumab and cetuximab to A431 EGFR-overexpressing cancer cells was equivalent. Surface Plasmon Resonance results were consistent with the FACS binding data.

Whether the equivalent binding of nimotuzumab and cetuximab to A431 cancer cells translates into similar levels of anti-tumor activity was examined by subcutaneously injecting SCID mice with A431 cells. As shown in Figure 3, nimotuzumab and cetuximab had identical anti-tumor activity in the xenograft study.

This translational research demonstrates that in clinical settings where cancer cells overexpress EGFR, equivalent anti-tumor activity is expected between nimotuzumab and cetuximab. However, the sparing of healthy tissues expressing low levels of EGFR will only result from the administration of nimotuzumab.

The current clinical development program for nimotuzumab is focused on EGFR-overexpressing malignancies and/or settings where nimotuzumab is combined with radiotherapy, which has been established to increase EGFR expression.^[2,3]

OBJECTIVES

- To examine whether the absence of severe rash and hypomagnesemia in clinical trials with nimotuzumab is a result of selective targeting of EGFR-overexpressing cancer cells by nimotuzumab and the antibody's lack of strong binding to epidermal and renal cells as compared to cetuximab.
- To compare anti-tumor activity of nimotuzumab and cetuximab against EGFR-overexpressing cells *in vivo*.

INTRODUCTION

Emerging evidence suggests that the ability of antibodies to bind bivalently (binding with both antibody arms to two targets simultaneously) is essential for maintaining prolonged drug residence in tumors and an important feature for inhibiting tumor cell proliferation.^[4-7,14] The formation of bivalent bonds is dependent on target density and antibody binding kinetics, such that increased receptor density on the cellular surface facilitates the formation of bivalent bonds. The strength of bivalent binding is characterized by avidity. With both antibody arms bound, the "bonus effect" occurs. This effect is due to decreased likelihood that both antibody arms will detach at the same time, which is required for the antibody to dissociate from the receptors. As a result, avidity is approximately equal to the square of affinity (strength of a monovalent bond). When receptor density is sufficiently high, bivalent binding is the most stable and preferred binding mode of antibodies to targets, provided epitopes permit such bond formation.

Nimotuzumab is an EGFR-targeting monoclonal antibody that has demonstrated anti-tumor activity in preclinical and clinical trials in the absence of severe side-effects commonly observed with other anti-EGFR antibodies, cetuximab and panitumumab. Nimotuzumab (Kd=10⁻⁸M) binds to the same or overlapping EGFR epitope as other antibodies and has been shown to inhibit EGFR activation, but has a lower affinity than either cetuximab (Kd=10⁻¹⁰M) or panitumumab (Kd=10⁻¹¹M).^[8-10]

EGFR is commonly overexpressed in various malignancies compared to normal levels of expression observed in organs such as skin and kidney. The severe rash and hypomagnesemia is believed to be caused by anti-EGFR antibodies binding to the receptor in tissues other than tumor. We investigated whether the decreased incidence of severe side-effects observed with nimotuzumab, while preserving similar levels of anti-tumor activity compared to cetuximab, is a consequence of nimotuzumab's optimal affinity for selective targeting of cancer cells overexpressing EGFR.

METHODS

- EGFR expression and FACS.** Immunoblot for total EGFR and actin for A431 cancer cell lines, human renal cortical epithelial cells (HRCE), human epidermal cells (HEC), and U1906 (EGFR expression negative control). The indicated cells were incubated with nimotuzumab or cetuximab mAbs followed by FITC-conjugated anti-human IgG. The cells were then labeled with a two-fold higher concentration of nimotuzumab or cetuximab monovalent antigen binding fragment (Fabs) (equimolar concentrations/the same binding sites) followed by FITC-conjugated anti-human IgG (Fab specific). The monovalent cetuximab and nimotuzumab fragments were generated by papain digestion of the antibodies as described previously by Fan *et al.*, 1993. For B and C, each point represents mean of triplicate wells; data were expressed as fluorescence mean intensity (FMI). All experiments were performed at least in triplicate.

RESULTS

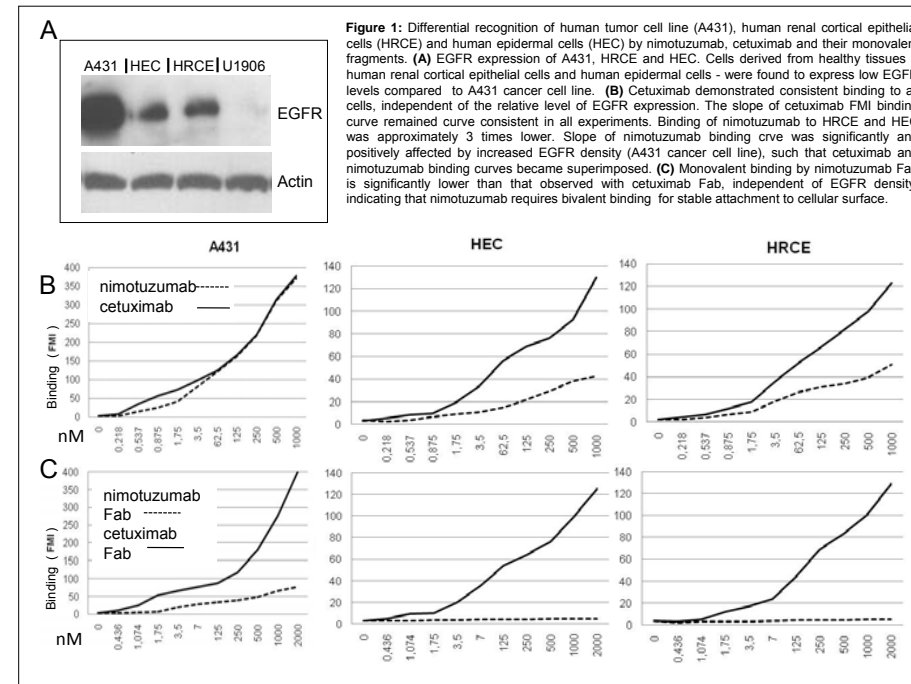


Figure 1: Differential recognition of human tumor cell line (A431), human renal cortical epithelial cells (HRCE) and human epidermal cells (HEC) by nimotuzumab, cetuximab and their monovalent fragments. (A) EGFR expression of A431, HRCE and HEC. Cells derived from healthy tissues – human renal cortical epithelial cells and human epidermal cells – were found to express low EGFR levels compared to A431 cancer cell line. (B) Cetuximab demonstrated consistent binding to all cells, independent of the relative level of EGFR expression. The slope of cetuximab FMI binding curve remained consistent in all experiments. Binding of nimotuzumab to HRCE and HEC was approximately 3 times lower. Slope of nimotuzumab binding curve was significantly and positively affected by increased EGFR density (A431 cancer cell line), such that cetuximab and nimotuzumab binding curves became superimposed. (C) Monovalent binding by nimotuzumab Fab is significantly lower than that observed with cetuximab Fab, independent of EGFR density, indicating that nimotuzumab requires bivalent binding for stable attachment to cellular surface.

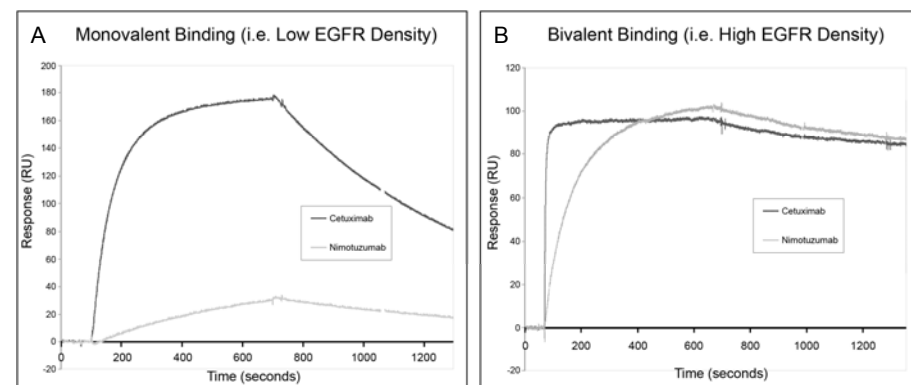


Figure 2: Examination of cetuximab and nimotuzumab binding kinetics using SPR (A) Under monovalent binding conditions (intended to represent binding to cells with low EGFR expression) the antibodies demonstrated significant difference in the level of accumulation, with very little binding occurring with nimotuzumab; ligand – anti-EGFR antibodies, analyte – 100nM EGFR monomer (B) Under bivalent binding conditions (intended to represent binding to cells with high EGFR expression), the antibodies behaved similarly and accumulated to the equivalent levels; ligand – Fc-EGFR chimera (dimer), analyte – 100nM anti-EGFR antibodies.

METHODS (continued)

- Surface Plasmon Resonance.** Antibody binding kinetics under monovalent (representing low EGFR expression) and bivalent binding conditions (representing high EGFR expression) were examined using Surface Plasmon Resonance (SPR; Biacore 3000). This technique allows us to study antibody binding kinetics without the need for enzymatic modification of the antibodies. 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 10min at 30µl/min followed by a 10min dissociation phase. Monovalent binding properties of the antibodies were examined by coupling cetuximab and nimotuzumab on the CM5 chip surface using the standard primary amine coupling reaction wizard. Increasing concentrations of EGFR monomer were flowed for 10min at 30µl/min followed by a 10min dissociation phase. Normalization of this chip was performed as per Pär Säfstén *et al.*, 2006. Efficient regeneration of both chip surfaces was achieved using two consecutive injections of 10µl of 50mM NaOH. All experiments were performed at least in duplicates. Representative data shown.
- In vivo experiments.** SCID mice, 6-8 weeks of age, were injected s.c. on day 0 with A431 cells (5x10⁶ cells/mouse). When tumors reached a mean size greater than 200 mm³, animals were randomized into one of the following treatment groups: control (PBS), nimotuzumab or cetuximab. Each group received 4 i.p. injections of either PBS or the indicated antibodies (1 mg/injection) every 48 hrs. Tumor volumes were evaluated every other day using the following formula: ½ larger diameter x (smaller diameter)².

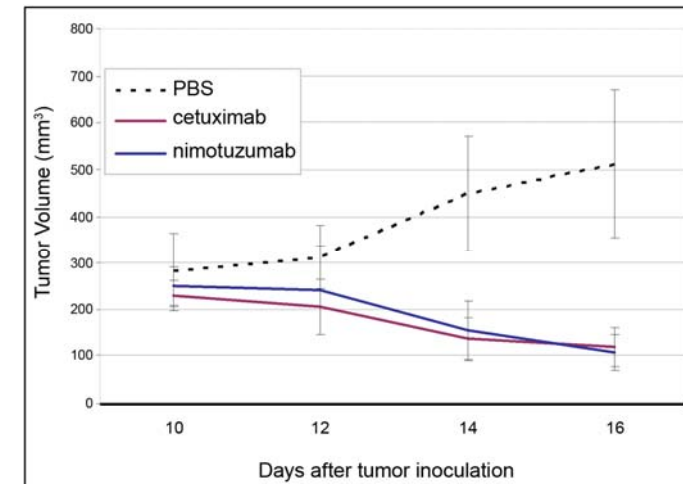


Figure 3: Effect of anti-EGFR mAbs nimotuzumab and cetuximab on the growth of established A431 xenografts in SCID mice. Mice received 4 i.p. injections (1 mg each) of either antibody. Similar tumor-growth inhibition observed with the antibodies.^[16]

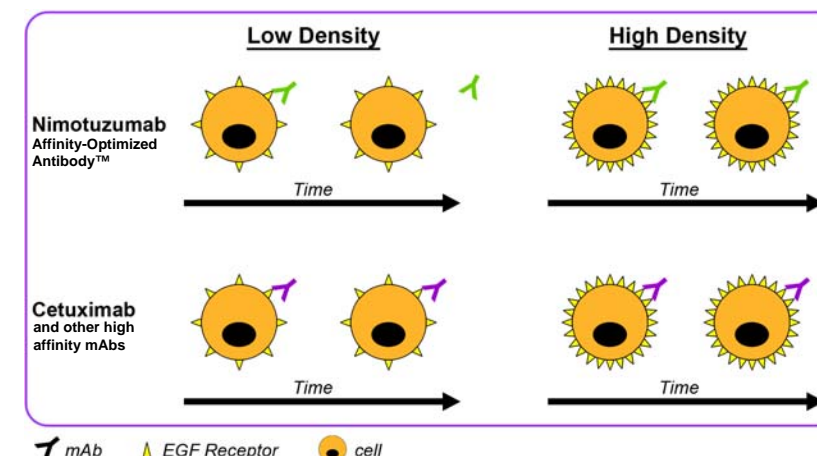


Figure 4: Schematic representation of nimotuzumab binding properties compared with higher affinity anti-EGFR antibodies as a function of EGFR density. In contrast to other anti-EGFR antibodies, under conditions of low EGFR density, nimotuzumab does not accumulate on the cellular surface, resulting in the sparing of healthy tissues such as skin, GI mucosa and kidneys. When EGFR density is high, bivalent attachment is the preferred and most stable binding confirmation for all antibodies causing the agents to behave similarly and accumulate to similar levels on tumor cells. As illustrated in Figure 3, the equivalent mode of binding of these agents results in equivalent anti-tumor activity against EGFR-overexpressing cells. This model explains the capacity of nimotuzumab to have similar anti-tumor activity as other EGFR inhibitors in absence of severe side-effects associated with targeting the EGFR pathway in healthy tissues.

DISCUSSION

- Marked differences in binding patterns of nimotuzumab and cetuximab to cells from healthy tissues (renal and skin) were observed.
- These differences were a function of lower EGFR density on healthy cells compared to A431 cancer cells (Figure 1A). The results demonstrate that cetuximab binds to normal cells because of high-affinity, monovalent interactions when EGFR is expressed at low levels, such as on normal cells. In contrast, monovalent binding of nimotuzumab is transient and results in a significantly reduced targeting of renal and epidermal cells. Both antibodies bind equivalently and bivalently when EGFR density is elevated (Figures 1, 2).
- When EGFR is overexpressed, such as on the surface of A431 cancer cells, nimotuzumab and cetuximab bind to cells with equivalent efficiency (Figures 1, 2), which translates into identical levels of anti-tumor activity (Figure 3).
- Figure 4 represents a schematic of the experimental observations. When EGFR expression is elevated, both antibodies behave similarly in that they bind with both arms (bivalently) and accumulate to a similar degree on the cell surface. When EGFR density is low, such as in healthy tissues, high-affinity antibodies continue to interact strongly with the receptors. In contrast, nimotuzumab targeting is antigen 'density-selective' and results in transient monovalent interaction thus avoiding targeting healthy tissues and the consequent severe toxicities.

DISCUSSION (continued)

- This translational research indicates that in clinical settings where cancer cells overexpress EGFR, equivalent anti-tumor activity is expected from nimotuzumab and cetuximab. However, the sparing of healthy tissues expressing low levels of EGFR, such as in skin and kidney, will only be achieved with the administration of nimotuzumab. This conclusion is supported by the results of randomized clinical trials with nimotuzumab in Squamous Cell Carcinoma of Head and Neck, a type of cancer known to overexpress EGFR.^[11]
- This study also indicates that unlike cetuximab, nimotuzumab will have limited activity against malignancies which do not overexpress EGFR. This conclusion is consistent with recently reported results of a nimotuzumab study in advanced colorectal cancer, a type of cancer known to express low EGFR density. In this study, a lower rate of response was observed with nimotuzumab than that reported for cetuximab in a similar patient population.^[12,13]
- The activity profile of nimotuzumab is similar to that of Herceptin®. This anti-Her2 antibody has a similar affinity constant to nimotuzumab, requires bivalency for activity and is only active against Her2-overexpressing malignancies.^[14,15]
- Transient monovalent binding by nimotuzumab to low-EGFR expressing tissue may lead to higher local tumor concentrations *in vivo* compared to high affinity antibodies. This is because EGFR is widely expressed in most tissues and as a result, these tissues may serve as a sink for high affinity antibodies and result in lower antibody concentration at the tumor site.
- In clinical indications where EGFR is not highly overexpressed, nimotuzumab is expected to have synergistic activity with therapies that increase EGFR expression, such as radiation-containing regimens.^[2,3] Encouraging early clinical data from trials with nimotuzumab combined with radiotherapy has been reported in NSCLC and brain metastasis from NSCLC.^[16,17]

CONCLUSIONS

- To the best of our knowledge, this is the first report to characterize the binding of nimotuzumab and cetuximab to human renal and epidermal cells. This translational research demonstrates that, unlike cetuximab, attachment of nimotuzumab requires bivalent binding. This property leads to nimotuzumab discriminating between cells that overexpress EGFR while minimizing interaction with tissues, such as kidney and skin, that express low EGFR levels.
 - The selective targeting of EGFR-overexpressing tumors by nimotuzumab is analogous to the selective targeting of HER2-overexpressing tumors observed with Herceptin®.
- These data, combined with rash being reported in patients treated with cetuximab independent of their KRAS status, indicate that severe toxicities of cetuximab should not be viewed as markers for clinical benefit of all EGFR-targeting antibodies. The severe toxicities arising with the administration of cetuximab are the result of that antibody's binding to all healthy tissues expressing EGFR.
- This research, combined with the accumulating clinical data on nimotuzumab, demonstrate that where tumors overexpress EGFR naturally or are stimulated to overexpress EGFR by radiation-containing regimens, nimotuzumab is expected to have similar efficacy to cetuximab and other high affinity antibodies.
- The current clinical development program for nimotuzumab is focused on indications where EGFR is overexpressed: either naturally, as a result of malignancy, or induced, as with radiation

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