

# Bivalent Binding Properties of Epidermal Growth Factor Receptor (EGFR) Targeted Monoclonal Antibodies: Factors Contributing to Differences in Observed Clinical Profiles

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## SUMMARY

Nimotuzumab is an EGFR-targeting antibody currently under investigation in multiple clinical trials. The antibody has demonstrated a unique clinical profile, where anti-tumor activity was observed in absence of severe skin, renal, GI mucosa and other toxicities commonly associated with EGFR-targeting antibodies, cetuximab and panitumumab.

Experimental observations presented here demonstrate that in contrast to other anti-EGFR antibodies, the intrinsic properties of nimotuzumab require bivalent binding (i.e. binding with both antibody arms to two targets simultaneously) for stable attachment to cellular surface, which leads to nimotuzumab selectively binding to cells that express moderate to high EGFR levels.

When EGFR density is low, such as on healthy tissues, cetuximab and panitumumab continue to interact strongly with the receptors. In contrast, nimotuzumab monovalent interaction is transient, thus sparing healthy tissues and avoiding the severe toxicities. When EGFR expression is moderate to high, all antibodies behave similarly in that they bind bivalently, the most stable mode of binding, and accumulate to similar degrees. Currently, there is no clinical evidence from studies with panitumumab and cetuximab that higher affinity (i.e. strength of monovalent interaction) leads to greater efficacy, though stronger monovalent binding clearly leads to higher toxicities. The binding properties of nimotuzumab may lead to improved *in-vivo* targeting of EGFR-overexpressing tumors, as compared with the other antibodies.

## INTRODUCTION

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.<sup>[1-4]</sup> 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.

The strength of bivalent binding is characterized by avidity. With both antibody arms bound, the "bonus effect" occurs. This effect is due to the 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 affinity (strength of a monovalent bond) squared. 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 binds to the same or overlapping EGFR epitope as other antibodies and has been shown to inhibit EGFR activation. Nimotuzumab has a lower affinity antibody as compared with cetuximab and panitumumab.

EGFR is commonly over-expressed in various malignancies as compared with the normal levels of expression observed in organs such as skin and kidney. The severe rash, hypomagnesaemia is believed to be caused by binding of the anti-EGFR antibodies to the receptor in tissues other than the tumor.

We investigated whether the decreased incidence of severe side-effects observed with nimotuzumab while preserving similar levels of anti-tumor activity as compared with cetuximab and panitumumab, is a consequence of different bivalent/monovalent binding profiles.

## OBJECTIVES

To examine whether the differences in clinical profiles between nimotuzumab and other EGFR-targeting antibodies may be a consequence of different bivalent/monovalent binding properties.

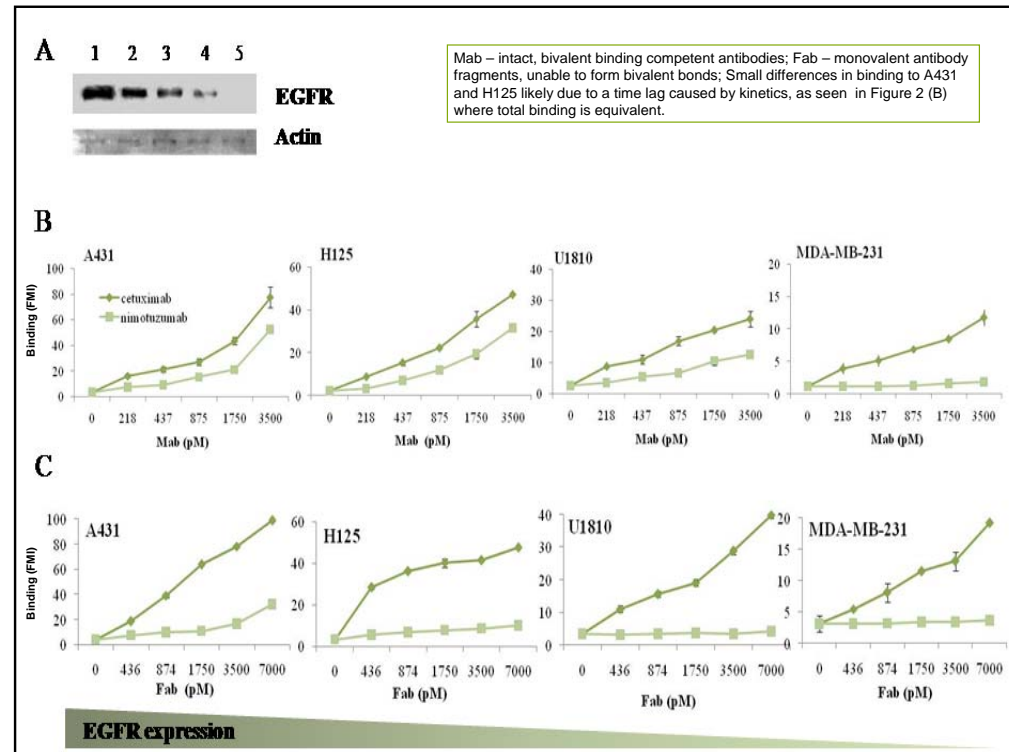
## METHODS

Immunoblot for total EGFR and actin were performed for A431, H125, U1810, MDA-MB-231 and U1906 cells. The cell lines were selected because of their varying levels of EGFR expression. The indicated cells were incubated with nimotuzumab or cetuximab mAbs followed by FITC-conjugated anti-human IgG. The cells were then labeled with 2-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 Mihaesco and Seligman, 1968. For B and C each point represents mean of triplicate wells; data were expressed as fluorescence mean intensity (FMI); and U1906 cell line was used as negative control of EGFR expression (data not shown). All experiments were performed at least in triplicates. Representative data are shown.

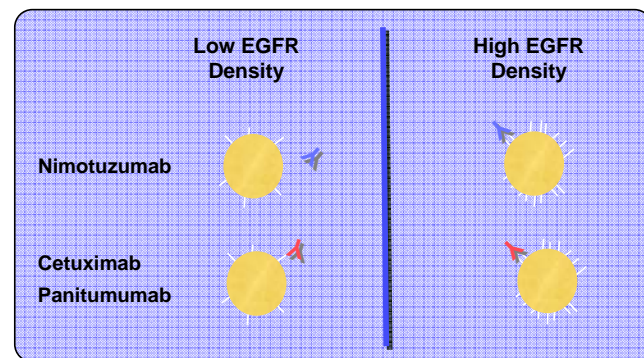
Antibody binding kinetics under monovalent (representing low EGFR expression) and bivalent binding conditions (representing high EGFR expression) were examined using Surface Plasmon Resonance (SPR; Biacore 3000). CM5 chip surface was coupled with FC-EGFR dimer using the standard primary amine coupling reaction wizard. Increasing concentrations of antibodies (nimotuzumab, panitumumab, and cetuximab) were flowed for 10min at 30µl/min followed by 10min dissociation phase. 1:1 Kinetics confirmed by fitting with BiaEvaluation software.

Monovalent binding properties of the antibodies were examined by coupling panitumumab, cetuximab, and nimotuzumab on CM5 chip surface using standard primary amine coupling reaction wizard. Increasing concentrations of EGFR monomer were flowed for 10min at 30µl/min followed by 10min dissociation phase. Normalization of this chip was performed as per Pär Säfssten *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 are shown.

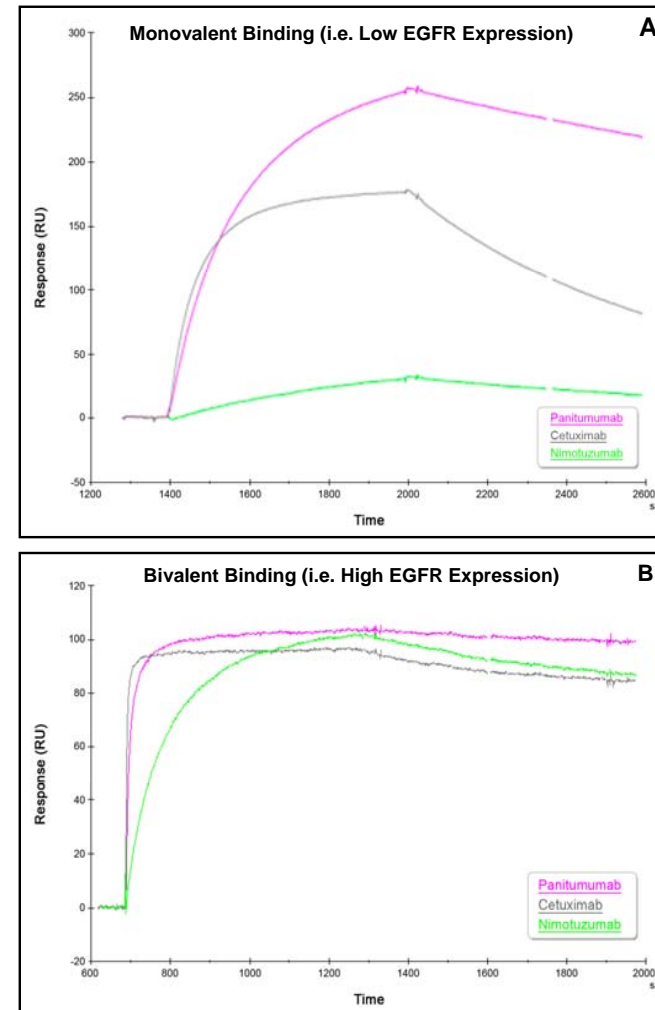
## RESULTS



**Figure 1:** Differential recognition of human tumor cell lines with different EGFR expression by nimotuzumab, cetuximab and their monovalent fragments. (A) Western Blot analysis showing different EGFR expression in different human tumor cell lines: A431 (1), H125 (2), U1810 (3), MDA-MB-231 (4) and U1906 (5) cells. U1906 cell line was used as negative control of EGFR expression (data not shown in B and C). (B) Nimotuzumab binding depends on EGFR density. Cetuximab 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 were parallel and close to each other. The small difference in the level of accumulation is likely the result of incubation time (see Figure 2(B) for details). (C) The level of monovalent binding by nimotuzumab Fab is significantly lower than that seen with cetuximab Fab, independently of EGFR density.



**Figure 3:** 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 cellular surface resulting in sparing of healthy tissues such as skin, GI mucosa, and kidneys. When EGFR density is high, bivalent attachment is the preferred, most stable binding confirmation for all antibodies causing the agents behave similarly and accumulating to similar levels on tumor cells. This model, supported by the present experimental results, elucidates the capacity of nimotuzumab to have similar anti-tumor activity with other EGFR inhibitors in absence of severe side-effects associated with targeting EGFR pathway in healthy tissues.



**Figure 2:** Examination of panitumumab (pink), cetuximab (grey) and nimotuzumab (green) binding kinetics using SPR (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 - 100nM 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 - 100nM anti-EGFR antibodies.

## DISCUSSION

- Marked differences in antibody binding patterns were observed as a function of EGFR density and binding mode (bivalent vs. monovalent). As shown in Figure 1 (B), cetuximab demonstrated consistent binding to all cell lines, independent of the 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 were parallel and close to each other.
- The binding of the monovalent fragments (Fab) is examined in Figure 1 (C). Cetuximab monovalent fragment 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.
- Antibody-EGFR interactions were then studied with SPR. The binding of cetuximab, panitumumab, and nimotuzumab was compared in real time under conditions allowing monovalent (i.e. low EGFR density) or bivalent binding (i.e. high EGFR density). The advantage of using SPR is the ability to gather data continuously in real time without the need for enzymatic modification of the antibodies. Consistent with FACS results, under monovalent conditions, Figure 1 (A), significant differences were observed between the levels of binding, with nimotuzumab demonstrating the lowest response amongst the antibodies.

## DISCUSSION (continued)

- Consistent with published literature, panitumumab had the strongest monovalent interaction with EGFR, followed by cetuximab. Interestingly, despite being administered half as frequently as cetuximab, panitumumab is reported to have higher frequency and severity of EGFR-related toxicities amongst the antibodies. At the moment there is no clinical evidence that higher affinity (i.e. strength of monovalent interaction) leads to greater clinical efficacy. In addition to causing severe toxicities, strong monovalent interactions with such large EGFR-expressing organs as skin may significantly impair *in-vivo* targeting of antibodies to EGFR-overexpressing malignancies.
- Consistent with FACS results, under conditions representing high EGFR density by allowing bivalent interactions (Figure 2 (B)), all anti-EGFR antibodies behaved remarkably similar, accumulating to equivalent levels.
- Figure 3 summarizes the results of the experimental observations in a schematic. 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 healthy tissues, cetuximab and panitumumab continue to interact strongly with the receptors. In contrast, nimotuzumab monovalent interaction is transient thus sparing healthy tissues and avoiding the severe toxicities.

## CONCLUSIONS

- Experimental observations presented here demonstrate that in contrast to other anti-EGFR antibodies, the intrinsic properties of nimotuzumab require bivalent binding for stable attachment, which leads to nimotuzumab selectively binding to cells that express moderate to high EGFR levels on the cellular surface.
  - The selective targeting of EGFR overexpressing tumors by nimotuzumab is analogous to observations with Herceptin®, where the antibody is only active against Her2-overexpressing malignancies.
- This distinct property of nimotuzumab provides a strong rationale for the unique clinical profile of this agent, specifically anti-tumor activity without severe skin, GI mucosa and renal toxicities, unlike other EGFR-targeting antibodies.
- Nimotuzumab is expected to have synergistic activity with agents that further increase EGFR activity, such as radiation containing regimens.<sup>[5]</sup>
- The selective binding of nimotuzumab to cells that over-express EGFR may result in superior targeting of EGFR-overexpressing tumors. At the moment there is no clinical evidence that higher affinity (i.e. strength of monovalent interaction) leads to greater clinical efficacy. There is clinical evidence from studies with panitumumab and cetuximab that stronger monovalent binding increases incidence and severity of toxicities. Compared to nimotuzumab at similar clinical doses, the strong monovalent interactions of the other antibodies with a large pool of normal EGFR-expressing cells is likely to lead to significantly impaired *in-vivo* targeting of these antibodies towards EGFR-overexpressing malignancies.

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