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Different antiproliferative effects of matuzumab and cetuximab in A431 cells are associated with persistent activity of the MAPK pathway

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ABSTRACT

Preclinical studies have shown the potential antitumour efficacy of monoclonal antibodies (MAbs) directed to the epidermal growth factor receptor (EGFR). In this report, we investigated the cytotoxic effects of the MAb matuzumab (EMD 72000) towards A431 cells and compared it to cetuximab. While cetuximab induced cell cycle arrest and inhibited A431 cell proliferation, matuzumab did not. Both MAbs inhibited growth factor induced EGFR, HER2 and AKT phosphorylation; however, only cetuximab inhibited ERK 1/2 phosphorylation. Taken together, the data indicate that each antibody may elicit different responses on EGFR downstream signalling pathways with a distinct impact on A431 cell line survival. When combined, MAbs synergistically inhibited cell proliferation and induced EGFR down-regulation with a strong inhibition of ERK1/2 and AKT phosphorylation. In addition, both MAbs efficiently inhibited VEGF expression and induced ADCC, highlighting their therapeutic potential *in vivo* when used either as a single agent or in combination.

1. Introduction

There has been substantial interest in the development of novel therapeutic agents for cancer treatment. The epidermal growth factor (EGF) receptor (EGFR) is one of the best characterised targets in cancer cells as it plays an important role in regulating cellular proliferation, differentiation and survival of normal epithelial tissues. Its overexpression in tumours is often correlated with poor prognosis, decreased survival and resistance to therapy.[1] Common cancer-related EGFR alterations are its overexpression, mutation/truncation and activation by excessive autocrine growth factor expression or heterodimerization with other ErbB family members.[2] EGFR deregulation contributes to proliferation, transformation, angiogenesis, invasion, metastasis and inhibition of apoptosis in cancer cells.

EGFR is a 170-kDa transmembrane glycoprotein with an extracellular domain that can bind EGF, transforming growth factor-alfa (TGF-α), amphiregulin (AR), betacellulin (BTC), heparin-binding-EGF (HB-EGF) and epiregulin (EPR). Along with HER2 (ErbB2/neu), HER3 (ErbB3) and HER4 (ErbB4), EGFR belongs to the ErbB/HER family of receptors. EGFR has a cytoplasmic domain with intrinsic protein-tyrosine kinase activity. Ligand binding induces autophosphorylation and

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the formation of homo or heterodimers with other members of the family. Receptor phosphorylation mediates the activation of the mitogen-activated protein (MAP) kinase, the phosphatidylinositol 3 kinase (PI3 kinase)/AKT, the phospholipase C gamma (PLC-γ)/protein kinase C (PKC) and the signal transducer and activator of transcription (STAT) pathways which regulate cell transformation, proliferation and survival.[3]

Anti-EGFR monoclonal antibodies (MAbs) are one of the most successful molecular-targeted drugs for cancer treatment. Cetuximab (Erbitux®), a chimeric human-murine MAb, binds EGFR with high affinity (K_D = 2.3 ± 0.5 nM), preventing ligand binding and receptor activation.[4] Cetuximab acts through the induction of receptor internalization and degradation,[5] upregulation of $p27^{kip1}$ and cell cycle arrest in G1,[6] enhanced apoptosis,[7] inhibition of angiogenesis[8,9] and induction of antibody-dependent cellular cytotoxicity (ADCC).[10,11]

The most common side-effect associated with cetuximab therapy is an acneiform rash that occurs in 70–80% of the patients due to immunogenic reactions because chimeric MAbs retain up to 30% of murine sequences. To make MAbs less antigenic and extend serum half-lives, murine antibodies have been humanised through the introduction of the antigen-binding region of the murine molecule into a human antibody backbone.[12]

Matuzumab, a humanised IgG1 derived from the murine precursor EMD 55900 (MAb 425), binds to EGFR with high affinity and induces ADCC in squamous cell carcinoma lines of the head and neck. Matuzumab and cetuximab have been evaluated in head-and-neck, colorectal, oesophageal, gastric, cervical and in non-small cell lung cancer.[7,11,13] Cetuximab is already integrated into therapy of colorectal and head and neck cancer. Several studies *in vitro* and *in vivo* have been published using A431 cells, and cetuximab alone or in combination with radiation and chemotherapy; however, there is a lack of information on the *in vitro* preclinical data for matuzumab.

In this report, we have characterised some of the molecular mechanisms that underlay matuzumab and cetuximab antitumour activity towards A431 cells *in vitro*. Our data show that even though matuzumab efficiently binds EGFR and blocks its phosphorylation, it is not as effective as cetuximab in inhibiting A431 cell proliferation as it fails to inhibit the MAPK pathway. However, the combination of MAbs synergistically reduced A431 cell proliferation, due to down-regulation of EGFR and inhibition of AKT and MAPK phosphorylation.

2. Materials and methods

2.1. Cell line

A431 cells were kindly provided by Dr. Giuseppe Giaccone (University Hospital Vrije Universiteit Amsterdam, The Netherlands).

2.2. Chemicals

Cetuximab and matuzumab monoclonal antibodies (MAbs) were generously provided by Merck KGaA (Darmstadt, Germany).

2.3. MTT and clonogenic assays (CAs)

For MTT, A431 cells were incubated with MAbs at different concentrations or incubated with 100 ug/mL of matuzumab or cetuximab in the presence/absence of PD98059 (25 uM). After 72 h, cells were incubated with a solution of tetrazolium reagent (MTT, Sigma) and processed as described in Meira et al.[14]

For CA, cells were incubated with matuzumab or cetuximab (10 and 100 ug/mL) for 72 h, washed and allowed to proliferate in fresh medium for 14 d. Colonies were stained with crystal violet as previously described by Ferreira et al.[15] For MAb combination experiments, matuzumab and cetuximab alone or combined at the same concentrations (50 ug/mL) were added and cells were maintained for 7 d before being stained with crystal violet. The number of colony-forming units in treated cultures was expressed as the surviving fraction (SF).

2.4. Drug interaction analysis

The cytotoxic effects of the combination of cetuximab and matuzumab were analysed according to Fischel et al.[16]

2.5. Cell cycle analysis

A431 cells were incubated alone or in the presence of 100 ug/mL of matuzumab or cetuximab, as described by Huang and Harari.[17] Cell cycle phase distribution was analysed by flow cytometry using propidium iodide (PI) staining, and the resulting DNA distributions were analysed on a Becton Dickinson FACScalibur.

2.6. Western blotting analysis

A431 cells were maintained in DMEM containing 10% of foetal bovine serum (FBS) v/v and prior to MAb treatments, cells were starved for 18 h in DMEM supplemented with 1% FBS v/v. This low serum concentration was selected so as to reduce exogenous growth factors while ensuring cell survival according to Fischel et al.[16] Prior to growth factor stimulation, cells were incubated for 4 h in serum-free medium (DMEM) in the presence of 100 ug/mL of matuzumab or cetuximab alone or incubated for 1 h with PD 98059, followed by incubation with 10 ng/mL of EGF as described by Raben et al.[18] Alternatively, A431 cells were incubated with the combination of cetuximab and matuzumab (50 ug/mL each) for 4 h in serum-free medium (DMEM) followed by incubation with 10 ng/mL of EGF. Membranes were incubated with antibodies against: phospho-AKT (Ser473), phospho-EGFR residues (Tyr 845, 992, 1045 and 1068), phospho-p44/42 extracellular signal-regulated kinase (ERK) 1/2 (Thr202/Tyr204), phospho-HER2 (Tyr877), EGFR, HER2, AKT, p44/42 ERK 1/2 (Cell Signalling Technology, Beverly, MA, USA). After incubation with secondary antibodies, immunoblots were detected using the enhanced chemiluminescence (ECL) reagent (GE Health Care, SP, Brazil), and bands were quantified with Labworks, version 4.6 (Bio-Rad, USA).

2.7. EGFR MAb binding

A431 cells were incubated either with a murine anti-EGFR MAb (EGFR purified mouse IgG_{2b} anti-human MAb), an isotype control mouse IgG_{2b}, an isotype control human IgG_1 (all from BD Pharmingen, BD Biosciences, San Diego, CA), cetuximab or matuzumab for 1 h on ice. After washing, an anti-mouse (Dako cytomation, Denmark) or anti-human (Caltag laboratories, USA) MAb was added to detect the primary antibodies. Samples were analysed on a Becton Dickinson FACScalibur using CELLQuest software (Becton Dickinson, CA).

2.8. In vitro ADCC assay

ADCC assay was performed with the kit Cyto Tox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI).[10] A431 cells were incubated alone or in the presence of 4 ug/mL of matuzumab or cetuximab for 4 h and exposed to peripheral blood mononuclear cells (PBMCs) at effector/target ratios (*E/T*) of 10:1 and 20:1 for 4 h. Specific cytolysis (ADCC) was calculated using the formula: $[ADCC\ (\%) = (A - B - C)/(D - B) \times 100]$, in which *A* represents experimental release; *B*, target cell spontaneous release; *C*, effector cell spontaneous release; and *D*, target cell maximum release.

2.9. Vascular endothelial growth factor (VEGF) mRNA and protein expression

A431 cells were incubated with 100 ug/mL of matuzumab or cetuximab for 24 h, total RNA was extracted, and used for reverse transcription (RT). Real time RT-PCR was performed with TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). *VEGF* and *GAPDH* mRNA levels were measured using Pre-developed TaqMan® Gene Expression Assays (Hs00173626_m1 and 4310884E, respectively). The relative expression level of *VEGF* mRNA was calculated using the comparative C_T method ($\Delta\Delta C_T$) and compared with untreated control cells as described elsewhere.[19]

VEGF protein concentration in the culture medium was determined using human VEGF ELISA Development Kit (Peprotech Inc., New Jersey, USA).

2.10. Statistical analysis

All experiments were done in triplicate and the values represent an average of at least three independent experiments. Statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software Incorporated, San Diego, CA). Quantitative experiments were analysed by Student's *t*-test. All *P* values resulted from the use of two-sided tests and were considered significant when <0.05.

3. Results

3.1. Cetuximab was more efficient in decreasing A431 cell viability in MTT and CA assays

Matuzumab treatment did not decrease A431 cell viability in short- or long-term cytotoxicity tests (MTT and CA), regardless of the concentration used (Fig. 1A–C). In contrast, cetuximab decreased A431 cell proliferation by 40% in MTT assays (Fig. 1A, $P < 0.05$) and, in CA, colonies were significantly smaller than controls and their number was reduced by 50% (Fig. 1B and C).

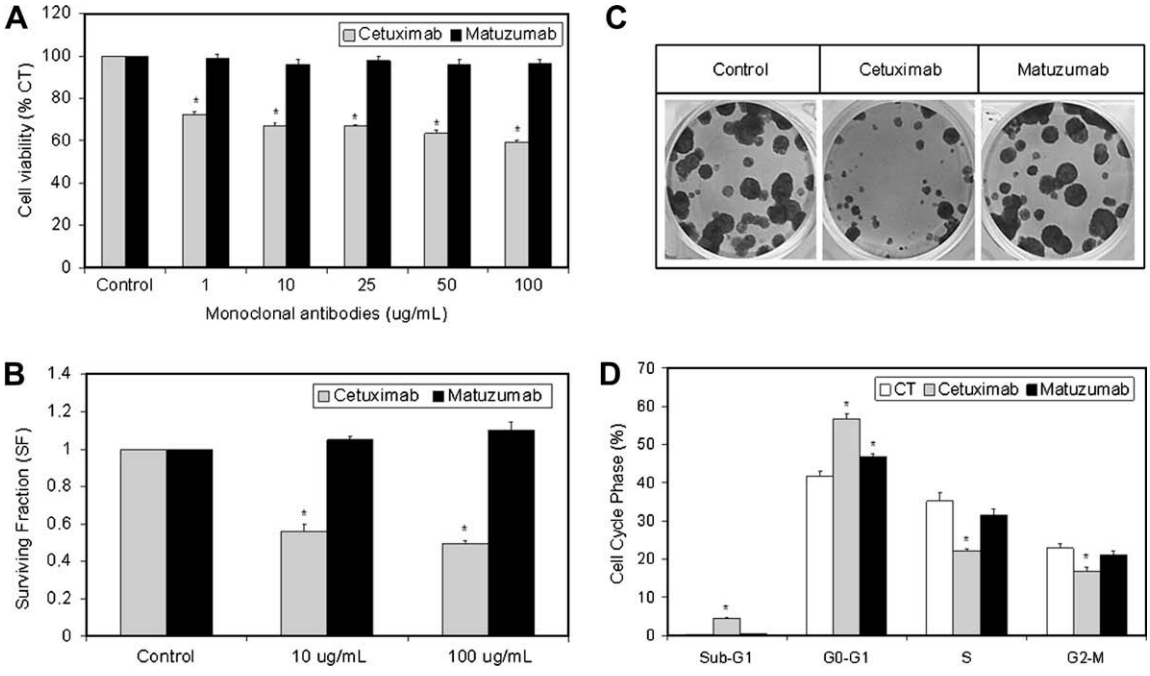

Fig. 1 – Effects of cetuximab or matuzumab on proliferation and cell cycle distribution of A431 cells. (A) MTT assay. (B) and (C) Clonogenic assays. (D) Cell cycle phase distribution by flow cytometry. Student's *t*-test *$P < 0.05$, compared to controls.

3.2. Cetuximab was more effective in inducing cell cycle arrest

An increase of 15.1 and 5.2% ($P < 0.05$) in cells at the G0/G1 phase of the cell cycle was observed after cetuximab or matuzumab treatment, respectively, with a simultaneous decrease in cells at the S and G2/M phases when compared to controls (CT) (Fig. 1D). Apoptotic cells undergoing DNA degradation are usually found in the sub-G1 phase of the cell cycle. Cetuximab induced an increase (4.5%) in this cell population after 24 h (Fig. 1D, $P < 0.05$), but only a small difference was detected after matuzumab treatment (0.4%) (Fig. 1D).

3.3. Both MAbs inhibited EGFR and HER2 protein phosphorylation

To explain the differences between MAbs in MTT and CA, we performed WB analysis to detect EGFR phosphorylation in cells that were treated with matuzumab and cetuximab alone or in the presence of EGF. As expected, EGF-induced EGFR phosphorylation, while both MAbs reduced it with similar efficiency (Fig. 2A). EGF and cetuximab also induced a slight decrease (19% and 14%, respectively) in the total amount of EGFR (Fig. 2A).

EGFR also interacts with HER2, forming heterodimers that are very potent in activating signalling transduction pathways.[3,20] Both MAbs inhibited EGF-induced HER2 phosphorylation with no change in total HER2 (Fig. 2B).

3.4. Matuzumab fails to inhibit ERK 1/2 phosphorylation

Both MAbs inhibited EGF-induced AKT phosphorylation (Fig. 2C). In contrast, cetuximab inhibited ERK 1/2 phosphorylation, while matuzumab did not (Fig. 2D).

In order to verify MAbs effects on MAPK intracellular signal transduction pathways, we used PD 98059, a specific MEK1/2 inhibitor, in combination with matuzumab or cetuximab in the presence or absence of EGF. This combination of PD 98059 and cetuximab completely abolished EGF-induced ERK 1/2 phosphorylation (Fig. 3A). However, this was not observed for the PD 98059 and matuzumab combination (Fig. 3A). MTT assays produced similar findings (Fig. 3B). These results show that even though matuzumab inhibits EGFR, HER2 and AKT phosphorylation, it was not able to inhibit MAPK cascade.

3.5. MAb combination down-regulates EGFR and reduces ERK1/2 and AKT phosphorylation

Recently it was demonstrated that treatment with anti-ErbB MAbs could enhance receptor down-regulation[21] and reduce cell proliferation. Furthermore, cetuximab and matuzumab can bind simultaneously to different regions in EGFR domain III.[22] When the combination of cetuximab with matuzumab (50 ug/mL each) was tested in CA they synergistically ($R = 0.27$) reduced the number and size of A431 colonies (Fig. 4A and B) when compared to each MAb alone ($P < 0.05$). Also, an increased down-regulation of EGFR was observed

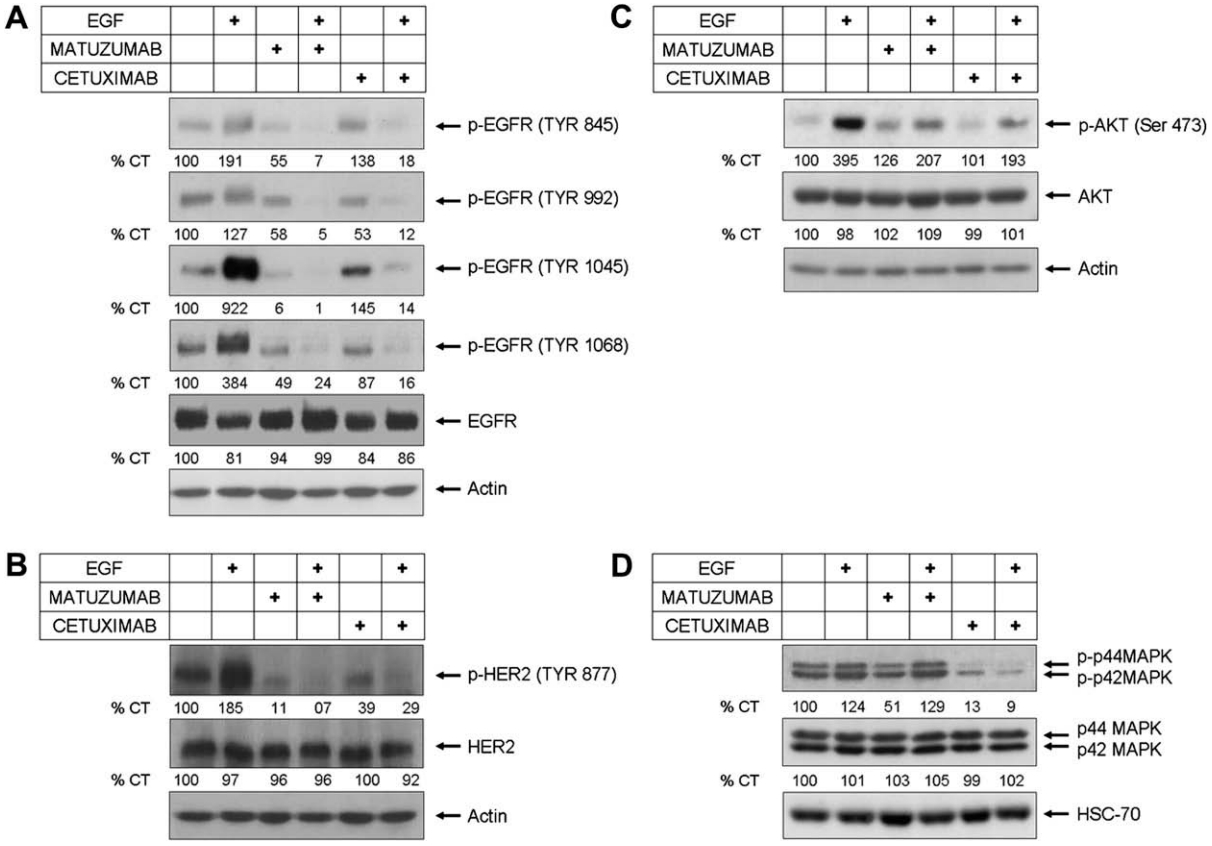

Fig. 2 – Effects of cetuximab or matuzumab alone on EGF-induced phosphorylation of (A) EGFR (Tyr845, Tyr992, Tyr1045 e Tyr1068). (B) HER2/*neu*. (C) AKT and (D) ERK 1/2 and total proteins on A431 cells by Western Blotting as described under material and methods.

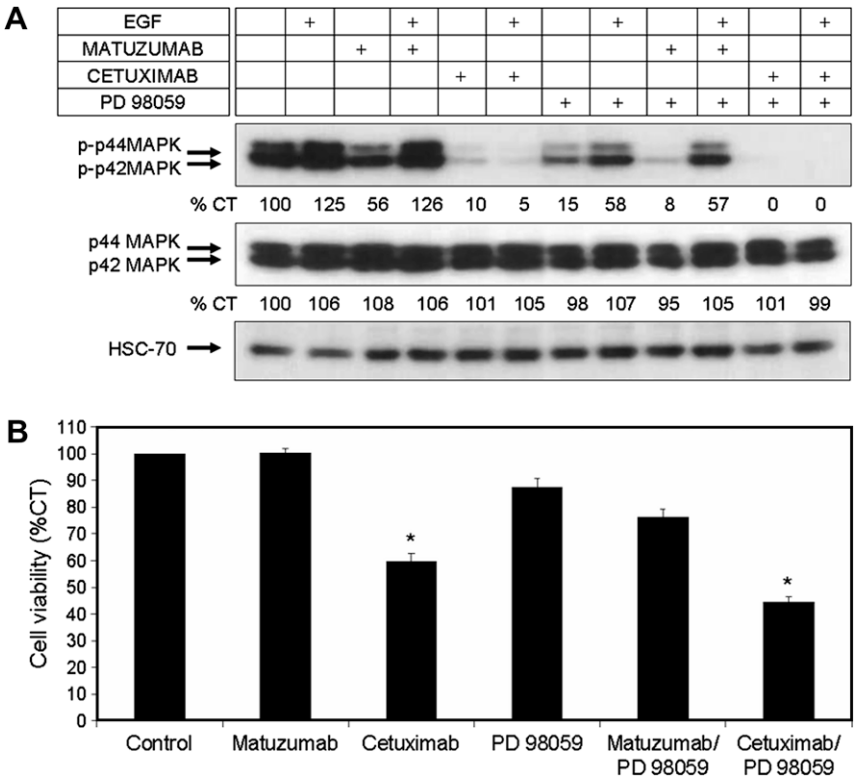

Fig. 3 – Effects of matuzumab and cetuximab alone or combined with PD98059 on A431 cells. (A) Western Blotting of p44/42 ERK 1/2 and phospho-p44/42 (ERK) 1/2. (B) MTT assay. Student's t-test $^{*}P < 0.05$, when compared to control cells.

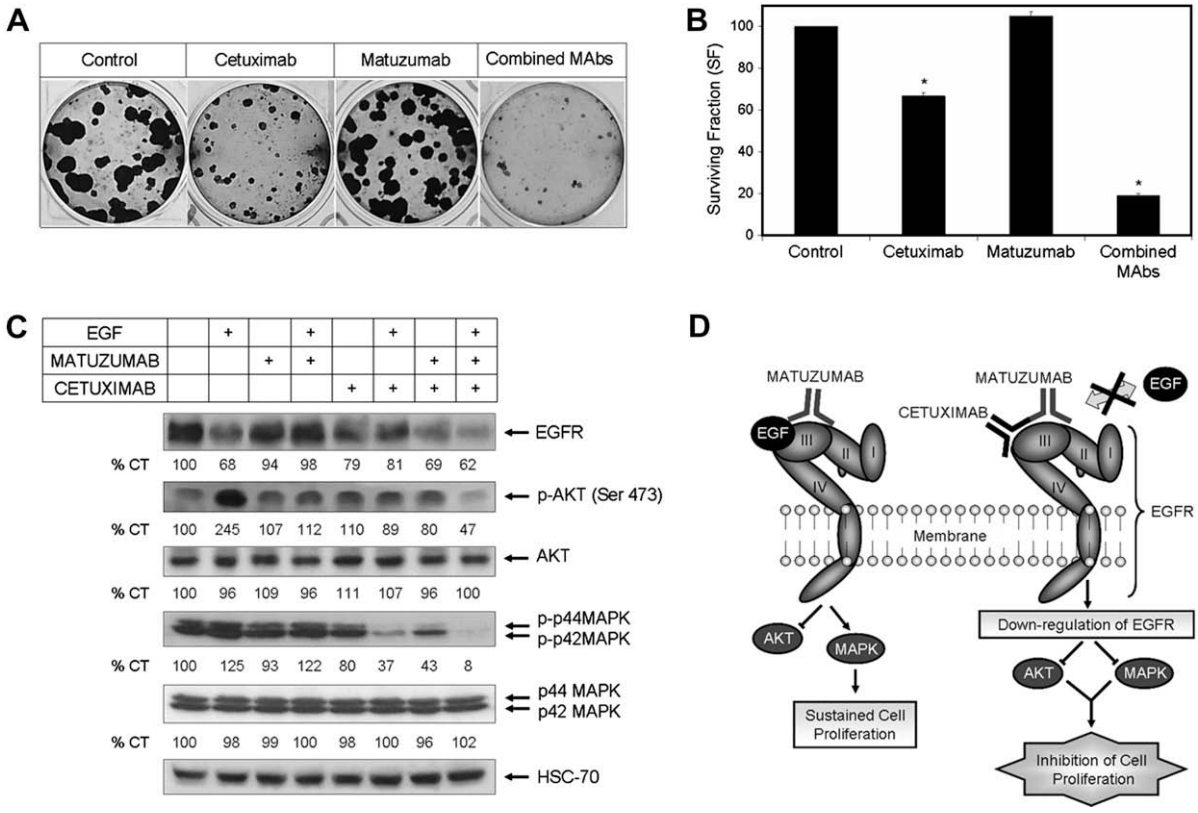

Fig. 4 – Effects of the combination of matuzumab and cetuximab on A431cells. (A) and (B) Clonogenic assays (CAs). (C) Western blotting for EGFR, AKT, ERK 1/2 total and phosphorylated. (D) Proposed model of the effects of the combination of matuzumab and cetuximab.

upon co-incubation with cetuximab and matuzumab (Fig. 4C). Likewise, we observed a strong reduction in ERK1/2 and AKT phosphorylation when MAbs were combined (Fig. 4C), with no changes in total protein. As EGFR phosphorylation activates the MAPK and AKT pathways, their simultaneous blockage may explain the synergistic effects obtained by this combination (Fig. 4D).

3.6. *Cetuximab and matuzumab efficiently bind to cell surface EGFR*

Anti-EGFR MAbs have different affinities and may differ in their capacity to bind cell surface EGFR. This could have implications for their use in cancer therapy, so we tested the MAbs ability to detect EGFR on A431 cell surface in comparison to another murine anti-EGFR MAb. As shown in Fig. 5A, matuzumab and cetuximab bind to EGFR in a similar fashion, displaying higher fluorescence intensity values than the commercial anti-EGFR antibody ($P < 0.05$).

3.7. *Cetuximab and matuzumab induced ADCC in vitro*

Inhibition of cancer cell proliferation can be achieved with the participation of immune and inflammatory mechanisms, such as MAb-induced ADCC.[10] Previous observations have demonstrated that cetuximab and matuzumab can induce ADCC in other cell lines[10,11,13,23], so we tested whether matuzumab could induce it in A431 cells. We observed that matuzumab induced ADCC in 8.9% and 20.8% of the cells at effector/target ratios of 10:1 and 20:1, respectively (Fig. 5B). Cetuximab, at these same effector/target ratios, behaved similarly, inducing ADCC in 12.9% and 28.4% of the cells.

3.8. *Cetuximab and matuzumab treatment down-regulated A431 VEGF mRNA and protein expression*

Anti-EGFR MAbs show suppressive effects on VEGF expression *in vitro* and *in vivo*.[9] In accord, both MAbs decreased *VEGF* mRNA expression by approximately 80% and protein content in the medium by 50% when compared with controls (Fig. 6A and B, respectively), suggesting that they have the potential of interfering with angiogenesis.

4. Discussion

Despite large clinical trials with anti-EGFR MAbs and the clinical use of cetuximab, several questions remain regarding

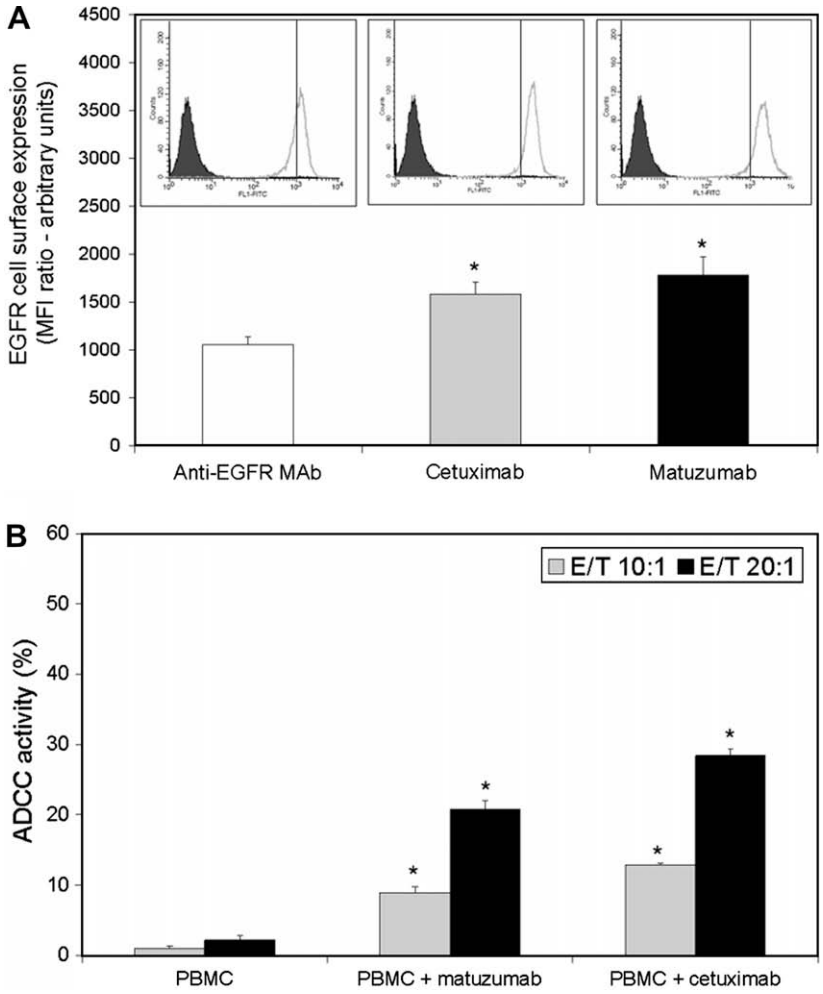

Fig. 5 – Effects of cetuximab or matuzumab on (A) EGFR cell surface expression and (B) ADCC in A431 cells as described under material and methods. Student's *t*-test *$P < 0.05$, when compared to PBMC alone.

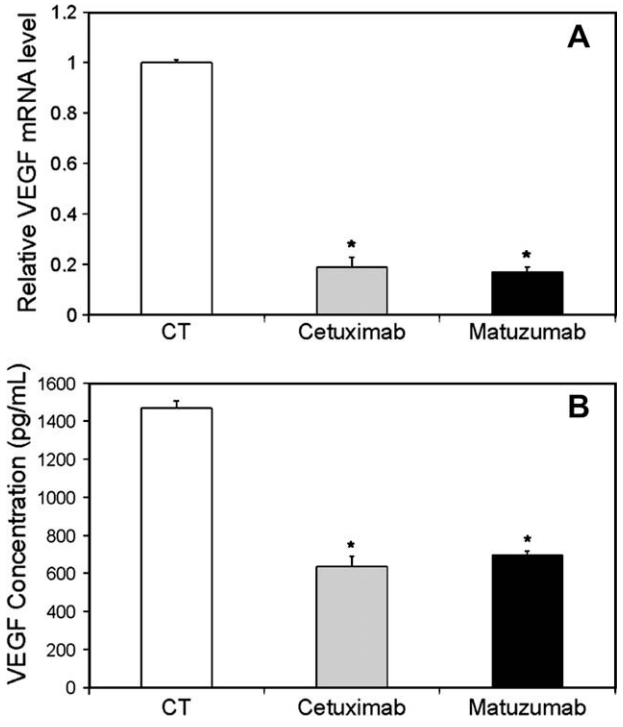

Fig. 6 – Effects of cetuximab or matuzumab on VEGF expression on A431 cells. (A) Relative *VEGF* mRNA level. (B) VEGF protein production detected by *ELISA*. Student's *t*-test *$P < 0.05$, when compared to controls.

their mechanisms of action. We investigated whether matuzumab and cetuximab could interfere with A431 cell proliferation *in vitro* and to which extent EGFR phosphorylation and downstream signalling pathways were inhibited. In addition, their ability to promote ADCC and modulate VEGF expression was also determined.

Previous studies indicated that the murine MAb 425, matuzumab precursor, was cytostatic, but not cytotoxic to A431 cells.[24] Accordingly, we observed that matuzumab did not decrease A431 cell viability when compared to cetuximab, although a slight increase in cell number at the G0/G1 phase of the cell cycle was noted.

Persistent signalling through downstream signalling pathways can contribute to EGFR inhibitor resistance.[7,18] In this context, the activation of EGFR leads to an increased activity of PI3 kinase, and the phosphorylation of the related protein-serine/threonine kinase AKT, with an impact on apoptosis, gene expression and cell proliferation. Sensitivity to EGFR inhibitors may also be affected by heterodimerization with HER2, with consequent induction of potent signalling.[3] We showed that both MAbs efficiently inhibited EGFR, HER2 and AKT phosphorylation, indicating their potential to inhibit downstream receptor signalling. Activated EGFR triggers the activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein (MAP) kinase pathway and our data showed that cetuximab, but not matuzumab, inhibited ERK 1/2 phosphorylation (Fig. 2D). A recent study, however, suggested that the antiproliferative effects of matuzumab and cetuximab on non-small cell lung cancer (NSCLC) cell lines relied on their ability to inhibit MAPK and AKT pathways[25] suggesting that matuzumab could inhibit ERK phosphorylation in other cell types.

A431 cells highly express EGFR (1.2×10^6 sites/cell) and largely depend on the EGFR/MAPK pathway for sustained proliferation.[7] Indeed, failure in disrupting this pathway could explain matuzumab's inability to decrease A431 viability. Similarly, it has been shown that resistance to gefitinib in breast carcinoma cells and to EGFR inhibitors (ZD1839 or cetuximab) in a panel of NSCLC cell lines was due to a persistent activation of both MAPK and AKT pathways.[7,26]

Inhibition of EGFR dimerization, activation and downstream signalling pathways may depend on how efficiently MAbs bind to the receptor.[4] Matuzumab binds to an epitope in EGFR domain III that does not interfere with ligand binding, while cetuximab's binding site overlaps the ligand binding region.[22] So, low affinity EGF binding can occur only in the presence of matuzumab.[22] This could partially explain A431 proliferation in the presence of matuzumab and EGF, suggesting that small differences in MAb binding could reflect major differences in EGFR signalling pathways.

Concomitant treatment with distinct anti-tyrosine kinase receptor MAbs can enhance receptor down-regulation and inhibition of EGFR and HER2 signalling *in vitro* and *in vivo*.[21] Therefore, the combination of cetuximab and matuzumab could be more effective due to a dual inhibition of the receptor, preventing ligand binding (cetuximab) and receptor dimerization (matuzumab).[22] Our data confirmed this hypothesis, since this combination induced synergistic antitumour effects on A431 cells.

Cetuximab, in combination with the receptor tyrosine kinase inhibitor lapatinib, leads to EGFR down-regulation and inhibition of downstream signalling pathways, reverting gefitinib resistance in EGFR mutated lung cancer cell lines.[27] In addition, the combination of anti-HER2 MAbs creates antibody-target complexes at the cell surface that favours HER2 internalization and down-regulation, inhibiting the MAPK pathway and tumour growth.[21] Accordingly, we observed that the combination of cetuximab and matuzumab, in the presence of EGF, synergised to induce EGFR down-regulation in A431 cells resulting in a greater inhibition of ERK1/2 and AKT phosphorylation (Fig. 4C) when compared to MAbs alone. MAPK and AKT cascade hyperactivation culminate in cell proliferation,[3,20] suggesting that the inhibition of these signalling pathways may play a central role in the effectiveness of dual-agent molecular targeting of EGFR. Based on these findings, we proposed a model of the combination of cetuximab and matuzumab on A431 cell proliferation (Fig. 4D).

Growth factors are expressed and secreted by the tumour cells themselves and stimulate cell proliferation.[3,20] Albanell et al. showed that when A431 cells are deprived of serum, proliferation is driven by endogenous (autocrine) ligand secretion, leading to higher basal levels of ERK1/2 phosphorylation. Indeed, they demonstrated that treatment with anti-EGFR compounds interferes with this autocrine stimulation thus inhibiting A431 cell growth.[28] Accordingly, cetuximab and, to a lower extent, matuzumab led to a reduction in the basal levels of ERK1/2 phosphorylation. In addition, the combination of MAbs synergistically suppressed basal ERK1/2 phosphorylation (Fig. 4C), indicating

the coexistence of shared and complementary mechanisms of action.

Additionally, we observed that EGFR/MAPK/AKT pathway activation was dominant for A431 cell survival and the strong reduction in p-ERK1/2 and p-AKT in EGFR dependent cells seen by the combination of two different MAbs correlated tightly with growth inhibition. Therefore, the inhibition of this pathway might be determinant for anti-EGFR therapeutic strategies. Altogether, we observed that the combination of matuzumab and cetuximab triggers synergistic effects on the inhibition of cell proliferation and EGFR downstream signalling pathways, suggesting that the combination of both MAbs could be beneficial for cancer therapy.

ADCC activity is an important anticancer mechanism induced by cetuximab and other MAbs, like trastuzumab and rituximab.[29] It has also been shown that cetuximab and matuzumab can induce ADCC *in vitro* in several cell lines.[10,23] Accordingly, we observed that matuzumab effectively induced ADCC in A431 cells suggesting that, *in vivo*, matuzumab treatment could enhance ADCC-mediated tumour cell death.

Anti-EGFR MAbs can also down-regulate VEGF expression.[8,9,13] We observed that both MAbs could reduce A431 *VEGF* mRNA and protein expression, indicating their potential role in the inhibition of angiogenesis *in vivo*. Recently, it has been shown that the down-regulation of hypoxia-inducible factor-1 alpha (HIF α) induced by cetuximab was not dependent on the inhibition of the MAPK pathway.[8,9] This could explain why matuzumab was as efficient as cetuximab in down-regulating VEGF expression in A431 cells.

To the best of our knowledge our study is the first to address the differences between cetuximab and matuzumab antiproliferative effects and their combinatorial ability to synergistically inhibit proliferation and downstream signalling pathways. This highlights the idea that a whole class-effect may not apply to anti-EGFR MAbs. We suggest that matuzumab's inability to inhibit A431 cell proliferation reflects the complexity of cells that heavily depend on the MAPK pathway and that may thereby develop resistance to molecular target therapy. In this context, this resistance mechanism could be overcome by the combination of anti-EGFR MAbs or even by different strategies that target the EGFR pathway.

We believe that the recognition of differential effects of cetuximab and matuzumab on tumour cells creates a new insight in our understanding of the antitumour mechanisms of anti-EGFR MAbs which may be helpful in directing and supporting a more rationale design of future clinical trials. We also propose that the combination of cetuximab and matuzumab could have potential clinical applications. We acknowledge, though, that our results are still limited to A431 cells and that for future studies it would be interesting to exploit different cell lines in order to draw more general conclusions about the effectiveness of matuzumab and cetuximab either alone or in combination.

Conflict of interest statement

None declared.

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REFERENCES

1. Newby JC, Johnston SR, Smith IE, Dowsett M. Expression of epidermal growth factor receptor and c-erbB2 during the development of tamoxifen resistance in human breast cancer. *Clin Cancer Res* 1997;**3**:1643–51.
2. Arteaga CL. Epidermal growth factor receptor dependence in human tumors: more than just expression? *Oncologist* 2002;**7**(Suppl. 4):31–9.
3. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;**2**:127–37.
4. Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* 2005;**7**:301–11.
5. Arnoletti JP, Buchsbaum DJ, Huang ZQ, et al. Mechanisms of resistance to erbitux (anti-epidermal growth factor receptor) combination therapy in pancreatic adenocarcinoma cells. *J Gastrointest Surg* 2004;**8**:960–9.
6. Harari PM, Huang SM. Combining EGFR inhibitors with radiation or chemotherapy: will preclinical studies predict clinical results? *Int J Radiat Oncol Biol Phys* 2004;**58**:976–83.
7. Janmaat ML, Kruyt FA, Rodriguez JA, Giaccone G. Response to epidermal growth factor receptor inhibitors in non-small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways. *Clin Cancer Res* 2003;**9**:2316–26.
8. Luwor RB, Lu Y, Li X, Mendelsohn J, Fan Z. The antiepidermal growth factor receptor monoclonal antibody cetuximab/C225 reduces hypoxia-inducible factor-1 alpha, leading to transcriptional inhibition of vascular endothelial growth factor expression. *Oncogene* 2005;**24**:4433–41.
9. Petit AM, Rak J, Hung MC, et al. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 1997;**151**:1523–30.
10. Kimura H, Sakai K, Arao T, Shimoyama T, Tamura T, Nishio K. Antibody-dependent cellular cytotoxicity of cetuximab against tumor cells with wild-type or mutant epidermal growth factor receptor. *Cancer Sci* 2007;**98**:1275–80.
11. Kawaguchi Y, Kono K, Mimura K, Sugai H, Akaike H, Fujii H. Cetuximab induce antibody-dependent cellular cytotoxicity against EGFR-expressing esophageal squamous cell carcinoma. *Int J Cancer* 2007;**120**:781–7.
12. Christiansen J, Rajasekaran AK. Biological impediments to monoclonal antibody-based cancer immunotherapy. *Mol Cancer Ther* 2004;**3**:1493–501.
13. Kim T. Technology evaluation: matuzumab, Merck KGaA. *Curr Opin Mol Ther* 2004;**6**:96–103.
14. Meira DD, Marinho-Carvalho MM, Teixeira CA, et al. Clotrimazole decreases human breast cancer cells viability through alterations in cytoskeleton-associated glycolytic enzymes. *Mol Genet Metab* 2005;**84**:354–62.

15. Ferreira CG, Span SW, Peters GJ, Kruyt FA, Giaccone G. Chemotherapy triggers apoptosis in a caspase-8-dependent and mitochondria-controlled manner in the non-small cell lung cancer cell line NCI-H460. *Cancer Res* 2000;**60**:7133–41.
16. Fischel JL, Formento P, Milano G. Epidermal growth factor receptor double targeting by a tyrosine kinase inhibitor (Iressa) and a monoclonal antibody (Cetuximab). Impact on cell growth and molecular factors 2. *Brit J Cancer* 2005;**92**:1063–8.
17. Huang SM, Harari PM. Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin Cancer Res* 2000;**6**:2166–74.
18. Raben D, Helfrich B, Chan DC, et al. The effects of cetuximab alone and in combination with radiation and/or chemotherapy in lung cancer. *Clin Cancer Res* 2005;**11**:795–805.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 2001;**25**:402–8.
20. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol* 2006;**7**:505–16.
21. Friedman LM, Rinon A, Schechter B, et al. Synergistic down-regulation of receptor tyrosine kinases by combinations of mAbs: implications for cancer immunotherapy5. *Proc Natl Acad Sci USA* 2005;**102**:1915–20.
22. Schmiedel J, Blaukat A, Li S, Knochel T, Ferguson KM. Matuzumab binding to EGFR prevents the conformational rearrangement required for dimerization 1. *Cancer Cell* 2008;**13**:365–73.
23. Bier H, Hoffmann T, Haas I, van Lierop A. Anti-(epidermal growth factor) receptor monoclonal antibodies for the induction of antibody-dependent cell-mediated cytotoxicity against squamous cell carcinoma lines of the head and neck. *Cancer Immunol Immun* 1998;**46**:167–73.
24. Rodeck U, Herlyn M, Herlyn D, et al. Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: immunologically mediated and effector cell-independent effects. *Cancer Res* 1987;**47**:3692–6.
25. Yoshida T, Okamoto I, Okabe T, et al. Matuzumab and cetuximab activate the epidermal growth factor receptor but fail to trigger downstream signaling by Akt or Erk. *Int J Cancer* 2008;**122**:1530–8.
26. Normanno N, De Luca A, Maiello MR, et al. The MEK/MAPK pathway is involved in the resistance of breast cancer cells to the EGFR tyrosine kinase inhibitor gefitinib. *J Cell Physiol* 2006;**207**:420–7.
27. Kim HP, Han SW, Kim SH, et al. Combined lapatinib and cetuximab enhance cytotoxicity against gefitinib-resistant lung cancer cells. *Mol Cancer Ther* 2008;**7**:607–15.
28. Albanell J, Codony-Servat J, Rojo F, et al. Activated extracellular signal-regulated kinases association with epidermal growth factor receptor/transforming growth factor alfa expression in head and neck squamous carcinoma and inhibition by anti-epidermal growth factor receptor treatments. *Cancer Res* 2001;**61**:6500–10.
29. Kurai J, Chikumi H, Hashimoto K, et al. Antibody-dependent cellular cytotoxicity mediated by cetuximab against lung cancer cell lines. *Clin Cancer Res* 2007;**13**:1552–61.