Clinical Cancer Research

See related commentary by Sabbatino and Ferrone, p. 958

GA201 (RG7160): A Novel, Humanized, Glycoengineered Anti-EGFR Antibody with Enhanced ADCC and Superior *In Vivo* Efficacy Compared with Cetuximab

Christian A. Gerdes[1], Valeria Gonzalez Nicolini[1], Sylvia Herter[1], Erwin van Puijenbroek[1], Sabine Lang[1], Michaela Roemmele[1], Ekkehard Moessner[1], Olivier Freytag[1], Thomas Friess[2], Carola H. Ries[2], Birgit Bossenmaier[2], Hans Joachim Mueller[2], and Pablo Umaña[1]

Abstract

Purpose: Anti-EGF receptor (EGFR) antibodies and small-molecule tyrosine kinase inhibitors have shown activity in epithelial tumors; however, agents that work by blocking the EGFR growth signal are ineffective when the oncogenic stimulus arises downstream, such as in tumors with *KRAS* mutations. Antibodies of the IgG_1 subclass can also kill tumor cells directly through antibody-dependent cell-mediated cytotoxicity (ADCC), and the efficacy of this is determined by the interaction of the Fc portion of the target cell–bound antibody and Fc receptors present on immune effector cells.

Experimental Design: We report the development of GA201, a novel anti-EGFR monoclonal antibody with enhanced ADCC properties. GA201 was derived by humanization of the rat ICR62 antibody. The Fc region of GA201 was glycoengineered to contain bisected, afucosylated carbohydrates for enhanced binding to FcγRIIIA.

Results: *In vitro* binding of GA201 to EGFR inhibited EGF ligand binding, EGFR/HER2 heterodimerization, downstream signaling, and cell proliferation to a similar extent as cetuximab. However, GA201 exhibited superior binding to both the low- and high-affinity variants of FcγRIIIA. This resulted in significantly enhanced induction of ADCC compared with cetuximab against both *KRAS*-wild-type and -mutant tumor cells lines. This enhanced ADCC translated into superior *in vivo* efficacy in a series of mouse xenograft models. Efficacy of GA201 was further increased when administered in combination with chemotherapy (irinotecan).

Conclusions: These data suggest that GA201 may be more effective than cetuximab in patients with EGFR-positive solid tumors and may also represent a first-in-class treatment of patients with *KRAS*-mutated tumors. *Clin Cancer Res; 19(5); 1126–38. ©2012 AACR.*

Introduction

The expression or activation of the EGF receptor (EGFR) has been shown in various epithelial malignancies including colorectal cancer (CRC), squamous cell carcinoma of the head and neck (HNSCC), and carcinomas of the pancreas, lung, cervix, renal cell, prostate, bladder, and breast (1). The level of EGFR overexpression correlates with poor prognosis of patients in many tumor types (1, 2). Thus, EGFR inhibitors encompassing both small molecules and antibodies have been developed for the treatment of cancer. The small-molecule EGFR tyrosine kinase inhibitors (TKI) erlotinib (Tarceva) and gefitinib (Iressa) have shown activity in multiple epithelial tumor types and have provided a scientific rationale for the development of EGFR antagonists as targeted therapeutics (3). These compounds reversibly bind to the ATP-binding site of the EGFR tyrosine kinase domain and inhibit autophosphorylation (4). Initial results with these molecules as monotherapy or in combination with chemotherapy in unselected populations were disappointing. It is now known that mutations in the *EGFR* gene alter the tumor phenotype and predict response to treatment, allowing the molecular selection of a subset of patients in which TKI are highly efficacious (5, 6).

The anti-EGFR monoclonal antibodies (mAb) cetuximab (Erbitux) and panitumumab (Vectibix) are established agents in the treatment of CRC and HNSCC. These agents have shown modest clinical efficacy in combination with chemotherapy in phase III trials (7–9). However, patients with CRC with *KRAS* mutations (30%–40% of patients) are unresponsive to cetuximab or panitumumab, when used as

Authors' Affiliations: [1]Roche Glycart AG, pRED, Schlieren, Switzerland; and [2]Roche Diagnostics GmbH, pRED, Penzberg, Germany

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Authors: Christian A. Gerdes, Roche Glycart AG, Wagistrasse 18 CH-8952 Schlieren, Switzerland. Phone: 41-44-755-6148; Fax: 41-44-755-6165; E-mail: christian.gerdes@roche.com; and Pablo Umaña, pablo.umana@roche.com

doi: 10.1158/1078-0432.CCR-12-0989

Translational Relevance

Anti-EGF receptor (EGFR) antibodies of the IgG_1 subtypes exert a therapeutic effect through two mechanisms of action: inhibition of signal transduction and direct cell killing by immune effector cells, such as natural killer cells and macrophages. GA201 is a glycoengineered anti-EGFR antibody of the IgG_1 subclass. GA201 inhibited EGFR signal transduction to a similar extent as cetuximab; however, glycoengineering of GA201 resulted in significantly enhanced induction of antibody-dependent cell-mediated cytotoxicity and *in vivo* efficacy in mouse xenograft models compared with both cetuximab and nonglycoengineered GA201. This superior efficacy of GA201 was seen in both KRAS-mutant and KRAS-wild-type mouse xenograft models and was further enhanced when combined with irinotecan chemotherapy. These data suggest that GA201 may be a more effective therapy than cetuximab in patients with EGFR-positive solid tumors and may represent a first-in-class treatment of patients with KRAS-mutated tumors.

monotherapy or in combination with chemotherapy (10–14). mAbs that target cell surface receptors can exert a therapeutic effect either by inhibiting the oncogenic growth signal (blocking ligand binding and/or receptor dimerization/activation) or through direct cell killing (15). Cell killing can be achieved by inducing apoptosis in the target cell or by recruiting cytotoxic immune effector cells such as macrophages, monocytes, and natural killer (NK) cells that can mediate antibody-dependent cell-mediated cytotoxicity (ADCC; refs. 16, 17). Inhibition of the EGFR growth signal will be ineffective in tumors that are *KRAS*-mutant, as the oncogenic stimulus in these cancers arises downstream of the EGFR receptor; however, these tumors should remain sensitive to cell killing by ADCC. As an antibody of the immunoglobulin G subclass 2 (IgG_2) class, panitumumab is devoid of macrophage- and NK cell–mediated ADCC (18). Cetuximab, being an antibody of the IgG_1 subclass can bind to FcγRIIIA and mediate ADCC to a certain extent (19–21). The lack of objective responses to cetuximab in *KRAS*-mutant patients indicates that a higher level of ADCC might be required to show a clinical benefit in this patient population.

The effective induction of ADCC is dependent on the interaction between the Fc region of the target cell–bound therapeutic antibody and the Fcγ receptors on the surface of immune effectors cells. This in turn is dependent on the antibody isotype, Fc sequence and glycosylation, and polymorphisms in the Fcγ receptors (18, 21–24). Antibodies of the IgG_1 isotype bind the Fcγ receptor with the highest affinity and are the preferred class of therapeutic antibodies for inducing ADCC (25). Glycoengineering of the Fc region of a therapeutic antibody to increase its binding affinity for FcγRIII can be further used to enhance its ADCC activity (26–29). Polymorphisms in FcγRIIA and FcγRIIIA affect the affinity with which mAbs bind to these effector cells (30). These polymorphisms have been shown to influence the efficacy of cetuximab; progression-free survival is significantly shorter in patients with the low-affinity variants compared with those homozygous for the high-affinity variants (23, 31, 32). Similar correlations between Fcγ receptor polymorphism and response have been reported for rituximab and trastuzumab (33, 34).

Our aim was to engineer a novel humanized anti-EGFR mAb with enhanced ADCC properties. Here, we report the development of GA201, an ADCC-optimized recombinant humanized anti-EGFR mAb of the IgG_1 isotype. Using GlycoMab technology (Roche Glycart AG), the Fc region of GA201 was glycoengineered to bear bisected, afucosylated carbohydrates. We hypothesized that the glycoengineering of GA201 would result in enhanced ADCC activity compared with cetuximab and that this would translate into superior efficacy in animal models of human cancer.

Materials and Methods

Production of GA201

Humanization of parental rat antibody ICR62. Humanization was conducted essentially by the complementarity-determining region (CDR) loop-grafting procedure (35). Rat ICR62 protein sequences [GenBank accession numbers GI:2300094 (heavy chain) and GI:2300096 (light chain)] were aligned to human germ-line sequences to identify human sequences with high sequence identity. The IGHV1-69*06 sequence (DP-88, GenBank: Z49804) and IGKV1-17 sequence (GenBank: X72808) were chosen as the framework acceptor sequences for the heavy and light chains, respectively. The 3 CDRs from the rodent heavy and light variable domains were grafted onto these acceptor frameworks. Because the framework 4 region is not part of the variable region of the germ line V gene, the alignment for that position was done individually. The JH6 sequence was chosen for the heavy chain, and the JK2 sequence was chosen for the light chain.

Glycoengineering of GA201. Glycoengineering of GA201 was conducted using GlycoMab technology (Roche Glycart AG) as described previously (36, 37). Briefly, the Fc region of GA201 was glycoengineered to bear bisected, afucosylated carbohydrates by coexpression with β1,4-N-acetylglucosaminyltransferase III and Golgi α-mannosidase II in Chinese hamster ovary cells. GA201 was produced using a batch-fed fermentation process and purified using protein A and ion-exchange column techniques.

Cell lines and antibodies

The human carcinoma cell lines A549, LS174-T, HT29, ACHN, Panc-1, and MDA-MB-231 were obtained from the American Type Culture Collection and, after expansion, deposited in the Roche Glycart internal cell bank. H460-M2 cells were obtained from Roche. Cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM)

(Gibco) containing 10% fetal calf serum (PAA Laboratories) at 37°C in a water-saturated atmosphere containing 5% CO_2.

NK-92 NK cells were genetically modified to create 2 cell lines expressing the high-(V158) and low-(F158) affinity variants of FcγRIIIA.

Commercial-grade cetuximab was obtained from Merck, panitumumab from Amgen, and the nonspecific total human IgG Redimune from CSL Behring.

Antibodies for Western blotting detection included polyclonal rabbit antibodies against human EGFR (#06-847, Millipore), HER2 (c-erB2 #A0485, Dako), and rabbit or mouse antibodies for mitogen-activated protein kinase (MAPK; 4695 or 9107, Cell Signaling Technology), $pMAPK^{Thr202/Tyr204}$ (#4370, Cell Signaling Technology), pS6 ribosomal $protein^{Ser235/Ser236}$ (#4858, Cell Signaling Technology), $pEGFR^{Tyr1173}$ (#1124-1, Epitomics), and β-actin (#4970, Cell Signaling Technology). Horseradish peroxidise–conjugated goat anti-rabbit and anti-mouse IgG (heavy and light chain) detection antibodies were used (#170-6515 and #170-6516, respectively, BioRad).

Inhibition of EGF ligand binding (ELISA)

Streptavidin-binding peptide-tagged EGFR-extra cellular domain (ECD) was incubated overnight at 4°C in streptavidin-coated microtiter plates (Roche Applied Science) to bind EGFR-ECD to the surface. After 3 washes with PBS +0.05% Tween, the plates were blocked with PBS containing 1% bovine serum albumin (BSA). Plates were then incubated with europium-labeled EGF (10 nmol/L) in the presence of increasing concentrations of GA201 or cetuximab (0.01–1,000 nmol/L) in Delfia Binding Buffer (PerkinElmer) for 1 hour at room temperature. Following 4 washes with Delfia Washing Buffer (PerkinElmer), the europium signal was measured using a multilabel reader (Victor Wallac) at 615 nm and IC_{50} values were calculated for both antibodies.

Western blotting

Western blot analysis of the inhibition of EGFR/HER2 heterodimer formation was conducted using Calu-3 non–small cell lung cancer (NSCLC) cells. Cells were cultured in minimum essential Eagle medium (PAN Biotech GmbH) at $\times 10^6$ cells per 6-well plate and were incubated with GA201 or cetuximab (both at 500 nmol/L formulated in histidine buffer) for 1 hour at 37°C. Histidine buffer and growth media alone were used as a control. Cells were washed with ice-cold PBS, lysed on ice for 10 minutes in Triton lysis buffer [150 mmol/L NaCl, 1.5 nmol/L $MgCl_2$, 100 mmol/L NaF, 10 nmol/L sodium pyrophosphate, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 10 μg/mL aprotinin, and 0.4 mmol/L othovanadat in 50 mmol/L HEPES buffer at pH 7.5] and centrifuged at maximum speed for 15 minutes at 4°C to remove cell debris. Heterodimers were isolated from the resulting supernatant by immunoprecipitation with 2 μg biotinylated anti-HER2 antibody (trastuzumab, Roche) overnight at 4°C. After washing, standard Western blotting was carried out and EGFR/HER2 heterodimers on immunoblots were visualized by enhanced chemilumiescence according to the manufacturer's recommendations (Amersham).

Inhibition of downstream signaling was investigated in A431 cells by Western blotting. Cells were cultured as spheroid structures in 96-well plates (1×10^4 cells per well) in the presence of GA201 or cetuximab at concentrations of 5 and 50 μg/mL for 48 hours. Following 2 washes with ice-cold PBS supplemented with 2 mmol/L Na_3VO_4, cells were lysed on ice for 10 minutes in radioimmunoprecipitation assay buffer (50 mmol/L Tris–HCl at pH 7.5 containing 150 mmol/L NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 10 mmol/L $Na_4P_2O_7$, 100 mmol/L NaF, and 2 mmol/L Na_3VO_4) supplemented with 1 μg/mL each of pepstatin, leupeptin, and aprotinin and 200 μg/mL PMSF. Cell lysates were cleared by centrifugation and standard immunoblotting was carried out using antibodies for EGFR, MAPK, $pMAPK^{Thr202/Tyr204}$, pS6 ribosomal $protein^{Ser235/Ser236}$, $pEGFR^{Try1173}$, and β-actin as a control.

Flow cytometry

The DNA content of isolated cell nuclei was determined by propidium iodide (PI) staining. A431 cells in culture were treated for 24, 48, or 72 hours with GA201, cetuximab (10 μg/mL each), or culture medium only (control). After removal of the supernatant, cells were washed once with PBS, detached using trypsin-EDTA (Gibco), and resuspended in the corresponding supernatant. Cells were pelleted at 1,000 rpm and 4°C for 5 minutes and resuspended in 500 μL DNA-staining buffer (0.1 mol/L Tris pH 7.4 containing 0.9% NaCl, 1 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 0.2% BSA, and 0.1% Nonidet P40). Cells were then incubated in 0.5 μL DNase-free RNAse A (Roche) at 500 μg/mL and 10 μL PI (Sigma) at 1 mg/mL for 30 minutes on ice in the dark, after which samples were analyzed using a FACScan (Becton-Dickinson) running CellQuest software. Doublets or aggregates of cell nuclei were excluded from the analysis by plotting FL3 peak width against FL3 peak height.

ADCC assays

ADCC was conducted using the Lactose Dehydrogenase Cytotoxicity Detection Kit (Roche) in accordance with manufacturer's instructions. Two different effector cell populations were used: FcγRIIIA-expressing NK-92 cells or human peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers. ADCC was conducted using effector:target (E:T) cell ratios ranging from 1:1 to 25:1 and incubation times ranging from 2 to 24 hours. Antibody concentrations of 1,500 ng/mL to 1 pg/mL were tested, and all assays were conducted in triplicate.

Mouse xenograft models

Severe combined immunodeficient (SCID)/beige (Taconics) and SCID human FcγRIIIA transgenic (Roche Glycart) female mice, ages 8 to 9 weeks were maintained under specific pathogen-free conditions with daily cycles of 12 hours light/dark according to guidelines (GV-SOLAS;

Federation of European Laboratory Animal Science Associations). Continuous health monitoring was carried out and the experimental study protocol was reviewed and approved by the Veterinary Department of Kanton Zurich (Switzerland). Food and water were provided *ad libitum*.

A series of orthotopic xenograft models reflecting the potential clinical indications for GA201 (lung, colorectal, renal, pancreas, and mammary tumors) were generated by inoculating human tumor cell lines into mice. Before injection, cells were harvested using trypsin-EDTA (Gibco), washed once in culture media, and resuspended in AIM V medium (Gibco). Orthotopic models were established using 1×10^6 cells (3×10^6 for the LS174-T CRC model). For the A549 and H460-M2 lung models, cells were injected intravenously. For other models, cells were injected directly into the target organ following laparotomy under deep general anesthesia (for the colorectal liver metastases models, the CRC cell lines LS174-T and HT29 were injected into the spleen).

Mice were randomized into different treatment groups (10 mice per group) and therapy started 7 or 14 days after tumor cell inoculation, when evidence of tumor growth was visible in the target organ of sacrificed scout animals. GA201, cetuximab, panitumumab, and the corresponding vehicle (control) were administered intravenously once weekly for 3 weeks at a dosage of 25 mg/kg.

Animals were assessed daily for clinical symptoms and adverse effects (general sickness, respiratory distress, and impaired motility). The termination criterion for sacrificing animals was sickness with locomotion impairment. Median overall survival (OS) was defined as the experimental day when at least 50% of animals in the group were sacrificed. Survival data were represented using Kaplan–Meier curves and differences in median OS between each treatment group were compared by means of the pairwise log-rank test. For the breast cancer MDA-MB-231 model, tumor growth control rates were investigated by measuring tumor volume [$^1/_2$ (length $\times$ (width)^2)] every 2 to 3 days, beginning on day 64. Therapy was started when tumor volume reached 200 mm^3.

Fluorescent immunohistochemistry

Detection of infiltrating macrophages and NK cells in xenograft biopsies was conducted according to standard immunohistochemical methods using 10-μm sections of frozen tumor tissue fixed in acetone. For detection of CD68-expressing cells, a rat anti-mouse CD68 antibody (MCA1957, Serotec) diluted 1:200 in PBS was used with an Alexa Fluor 568–conjugated goat anti-rat IgG secondary antibody (#A-11077, Invitrogen) diluted 1:400 in PBS. For NK-p46–expressing cells, a goat anti-mouse NKp46 antibody (#AF2225, R&D Systems) at 1 μg/L in PBS was used with a DyLight 488–conjugated rabbit anti-goat IgG secondary antibody (#305-486-047, Jackson). Before incubation of primary or secondary antibodies, sections were blocked with the relevant 10% serum-blocking solution (goat or rabbit, depending on the secondary antibody used). Antibodies of the same isotype as the primary antibodies (Serotec) were used as negative controls in consecutive slides to detect nonspecific binding.

Results

Engineering and characterization of GA201

GA201 was derived by humanization of the parental rat IgG_{2b} antibody ICR62 (38). The *in vitro* and *in vivo* biologic activity of ICR62 has been described previously: ICR62 inhibits EGF binding, EGFR-mediated signaling, and growth of human EGFR-overexpressing tumor xenografts in mouse models (leading to complete tumor regression in some cases; ref. 38). In addition, ICR62 has been shown to bind effectively to cells expressing wild-type EGFR and EGFR variant III (39). The humanized GA201 construct showed identical EGFR-binding behavior as the parental rat antibody. No amino acids outside of the CDR regions were needed to be of rodent origin (back mutation); therefore, the framework of the heavy and light chains was fully human.

GA201 inhibits EGFR binding, EGFR/HER2 receptor heterodimerization and downstream signaling *in vitro*

GA201 bound with high affinity to domain III of the ECD of human EGFR. GA201 neither competed for, nor prevented, the binding of cetuximab and panitumumab indicating that GA201 binds to a different epitope. GA201 bound to EGFR-overexpressing A431 human epidermoid carcinoma cells with an apparent K_D value of 1 to 2 nmol/L (as determined by fluorescence-activated cell sorting-based binding assays). Analogous binding assays with recombinant human EGFR transiently transfected into Chinese hamster ovary cells showed a 2- to 3-fold lower affinity of GA201 compared with cetuximab. When using Fab fragments instead of whole bivalent antibodies, GA201 monovalent Fab fragments bound to the ECD of human EGFR with a K_D value of 4 nmol/L. ELISA analysis of immobilized EGFR-ECD identified a concentration-dependent inhibition of EGF ligand binding by GA201 (Fig. 1A). Both GA201 and cetuximab inhibited the binding of EGF to the EGFR-ECD with an IC_{50} of about 0.33 nmol/L.

Dimerization of EGFR with other members of the HER family is important for downstream signaling (40). Among the potential heterodimerization partners of EGFR, HER2 is the preferred one as HER2 is present in an open conformation, is ligand independent, and provides numerous phosphotyrosine residues that are activated after successful receptor heterodimerization (41). The effect of GA201 on EGFR heterodimerization with HER2 was investigated using Calu-3 NSCLC cells, which moderately express EGFR and overexpress HER2 on the cell surface. Compared with control, treatment with 500 nmol/L GA201 effectively inhibited the formation of EGFR/HER2 heterodimers in Calu-3 cells (Fig. 1B), and again the magnitude of this effect appeared similar to that of cetuximab.

Inhibition of EGF-binding and EGFR dimerization translated into inhibition of downstream signaling. At 5 and 50 μg/mL, GA201 inhibited the phosphorylation of EGFR and

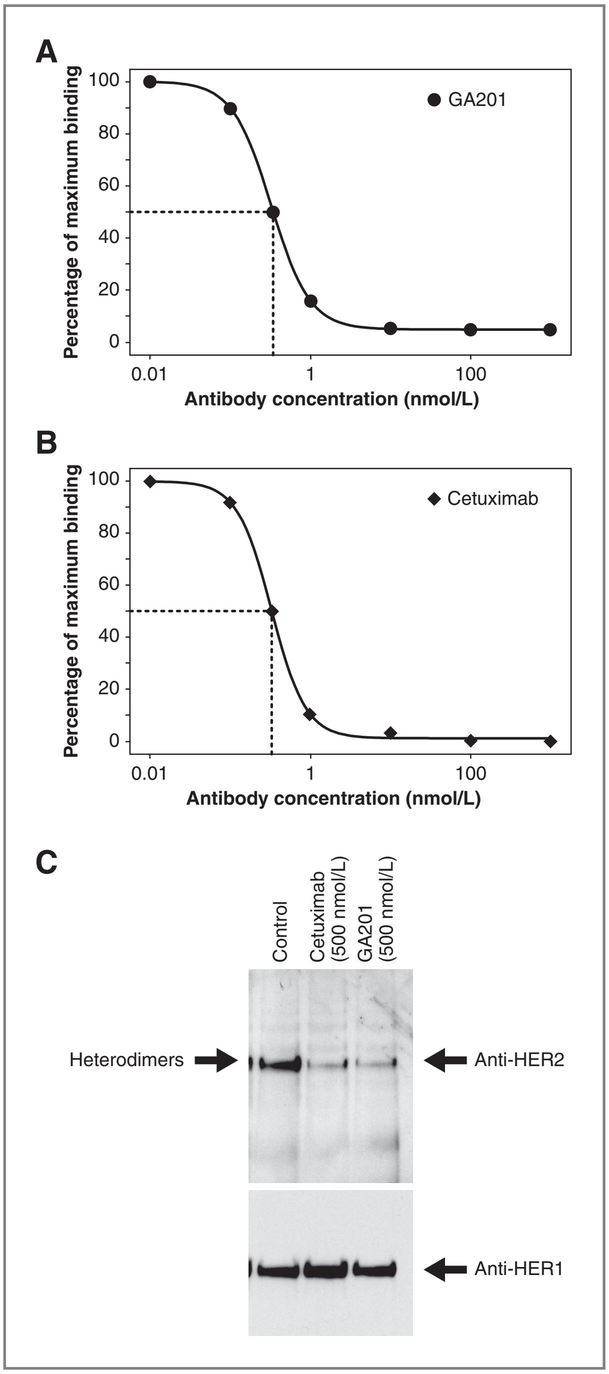

Figure 1. Inhibition of EGF ligand binding and EGFR/HER2 heterodimerization. A and B, ELISAs were conducted with immobilized EGFR-ECD in which the binding of europium-labeled EGF in the presence of increasing concentrations of GA201 or cetuximab was measured. The data show that both antibodies inhibit the binding of EGF to the EGFR-ECD with an IC_{50} of about 0.33 nmol/L. C, Calu-3 human NSCLC cells with moderate surface expression of EGFR and overexpression of HER2 were seeded and treated with 500 nmol/L GA201 or cetuximab or control. After harvesting, cells were lysed and EGFR/HER2 heterodimers were visualized by immunoblotting. Compared with control, treatment of Calu-3 cells with GA201 and cetuximab significantly inhibited the formation of EGFR/HER2 heterodimers. Data are representative of 2 independent experiments with similar results.

other key downstream signaling molecules (including MAPK and S6) to a similar extent as cetuximab (Fig. 2A).

To determine whether signaling inhibition mediated by GA201 resulted in reduced proliferation or induction of apoptosis, we conducted a flow cytometry–based cell-cycle analysis. In this assay, nuclear DNA is quantified allowing analysis of cell populations in the G_1 or S–G_2 phase of the cell cycle as well as fragmented nuclei in the sub-G_1 fraction (42). A431 cells were incubated with different concentrations of GA201 or cetuximab and analyzed after 24, 48, and 72 hours. After 24 hours, an increase in the sub-G_1 population was already apparent with both GA201 (26% at 10 μg/mL GA201) and cetuximab (32%) compared with control cells treated with culture medium only (13%; Fig. 2B). The population of sub-G_1 cells increased in a time-dependent manner. Moreover, both the G_1 and S–G_2 subpopulations decreased, indicating that DNA fragmentation was occurring in all phases of the cell cycle. GA201 treatment did not affect cell-cycle progression.

Glycoengineering of the Fc region

Glycoengineering of GA201 resulted in bisected, afucosylated Fc-region carbohydrates and a concomitant increase in the affinity for FcγRIIIA compared with the parental ICR62 antibody. Using surface plasmon resonance, the affinity of glycoengineered GA201 (85% afucosylation) for the human high-affinity FcγRIIIA-V158 variant was enhanced approximately 50-fold compared with cetuximab (average K_D values for GA201 and cetuximab were 25 nmol/L and 1,280 nmol/L, respectively). Affinity for the low-affinity FcγRIIIA-F158 variant was enhanced approximately 27-fold (average K_D values for GA201 and cetuximab were 240 nmol/L and 2,050 nmol/L, respectively). Thus, glycoengineering resulted in a significantly increased affinity of GA201 for both the low- and high-affinity human FcγRIIIA variants compared with cetuximab. In addition, glycoengineering did not affect binding to the inhibitory human FcγRIIB (43).

GA201 exhibits superior *in vitro* ADCC compared with cetuximab in the presence of both FcγRIIIA high- and low-affinity human NK cells

To investigate whether the enhanced affinity of GA201 for the low- and high-affinity FcγRIIIA variants compared with cetuximab translated into enhanced cell killing, a series of ADCC assays were conducted. GA201 exhibited superior ADCC to cetuximab at all E:T ratios tested. Optimal results were obtained using NK-92 cells as effectors at an E:T ratio of 3:1 with a 2-hour incubation time and using human PBMCs at an E:T ratio of 25:1 with a 4-hour incubation time (Fig. 3). Data for the MKN45 cell line at different E:T ratios are presented in Supplementary Fig. S1. Using *KRAS*-wild-type, EGFR-overexpressing A431 cells as target cells and human NK-92 (V158) cells (which express the high-affinity FcγRIIIA) as effector cells at an E:T ratio of 3:1, GA201 was approximately 2-fold more efficacious than the same concentration of cetuximab at inducing ADCC (Fig. 3A). In the presence of physiologic concentrations of competing

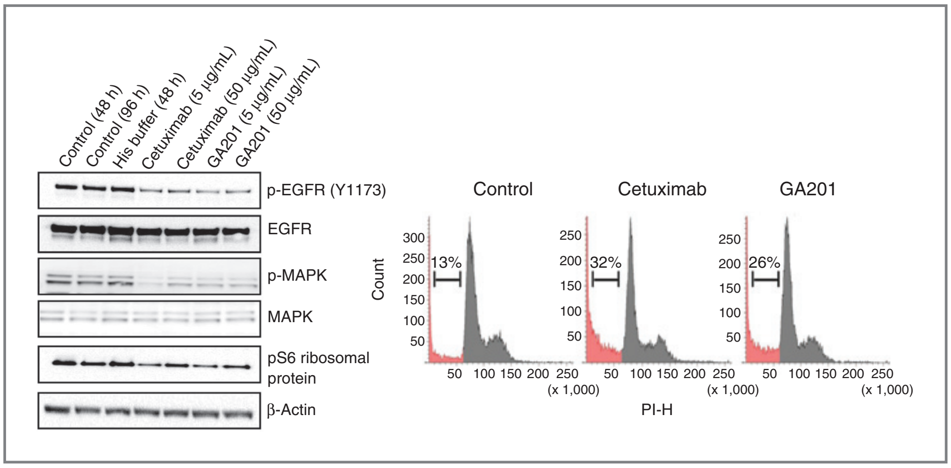

Figure 2. Inhibition of EGFR signaling and induction of nuclear fragmentation in A431 cells. A, A431 tumor cell spheroids were cultured in DMEM supplemented with 10% FBS and 4 mmol/L L-glutamine. Following a 48-hour treatment with GA201 or cetuximab, cells were washed, lysed, and subjected to Western blot analyses. GA201 and cetuximab show comparable inhibition of EGFR signaling in A431 cells indicated by reduced pEGFR, pMAPK, and pS6 levels. Data are representative of 2 independent experiments. B, A431 cells treated with EGFR-neutralizing antibodies for 72 hours, after which cell nuclei were isolated and stained with the DNA dye PI. Apoptotic cells with sub-G_1 chromosomal DNA content are expressed as a percentage of total gated cells. Treatment with both GA201 and cetuximab resulted in an increase in the sub-G_1 population compared with control. The results of this single experiment were confirmed by Hoeschst/bromodeoxyuridine staining (data not shown).

nonspecific total human IgG (5 mg/mL Redimune), GA201 continued to exhibit significant ADCC activity, whereas the ADCC activity of cetuximab was virtually abolished. When human NK-92 (F158) cells (low-affinity FcγRIIIA) were used as effector cells, GA201 was approximately 35-fold more potent than cetuximab at inducing ADCC (Fig. 3B).

GA201 also exhibited superior *in vitro* efficacy over cetuximab when A549 cells (low levels of EGFR, mutant *KRAS*) were used as target cells and high-affinity NK-92 (V158) cells were used as effectors. A nonglycoengineered version of GA201 ($GA201_{wt}$) was also investigated. In this setting of low EGFR expression, *KRAS*-mutant target cells, and low affinity FcγRIIIA on effector cells, GA201 continued to show a high level of ADCC, whereas the ADCC activity of cetuximab and $GA201_{wt}$ was virtually nonexistent (Fig. 3C). In the presence of nonspecific total human IgG, GA201 retained some ADCC activity at higher antibody concentrations.

When human PBMCs were used as effector cells, GA201 again proved to be superior to both cetuximab and $GA201_{wt}$ in inducing ADCC against a variety of tumor cell lines (Fig. 3D–E).

In vivo efficacy of GA201 in tumor xenograft models with different levels of EGFR expression

GA201 showed superior *in vivo* efficacy compared with cetuximab in a series of mouse xenograft models bearing murine monocytes, macrophages and/or NK cells as immune effector cells displaying murine FcγRIV and/or human FcγRIIIA. In mice, the homolog receptor to the human FcγRIIIA is the murine FcγRIV, which is present on murine monocytes and macrophages but not on murine NK cells, as is the case with human FcγRIIIA. Important contributions to efficacy of antitumor antibodies via FcγR-dependent immune effector functions have previously been reported for various antibodies in murine models (17, 25, 44).

Initial modeling was conducted using SCID/beige mice. These mice bear only monocytes and macrophages as fully active leukocytes capable of mediating FcγRIV-dependent immune effector functions. Consequently, the SCID/beige mouse model can only show the antitumoral effect of ADCC (and/or FcγRIV-triggered antibody-dependent cellular phagocytosis and cytokine release) mediated by monocytes and macrophages as effectors (i.e., no NK cell contribution) and inhibition of EGFR signaling inhibition via antibody-mediated receptor blockade. Additional modeling was conducted in SCID human FcγRIIIA transgenic mice generated in-house. These mice bear both murine FcγRIV-positive murine monocytes and macrophages and human FcγRIIIA-positive murine NK cells as effectors (approximately 80% of murine NK cells express human FcγRIIIA), allowing a more realistic representation of GA201 efficacy via NK cell–mediated ADCC.

GA201 efficacy was first evaluated in a human lung adenocarcinoma orthotopic xenograft model in which A549 cells (medium/low EGFR expression, *KRAS*-mutant) were injected intravenously into SCID/beige mice (bearing active FcγRIV monocytes and macrophages as effectors). Following therapeutic treatment with antibodies (25 mg/kg), median OS was significantly increased in animals receiving GA201 compared with cetuximab ($P = 0.028$), panitumumab ($P < 0.001$), or vehicle control ($P < 0.001$; Fig. 4A). Similar survival was observed in animals treated with a single dose of either 25 or 125 mg/kg GA201 with no dose-dependent efficacy observed (data not shown). Using the same model, median OS was also significantly greater with a single intraperitoneal injection of GA201 (25 mg/kg)

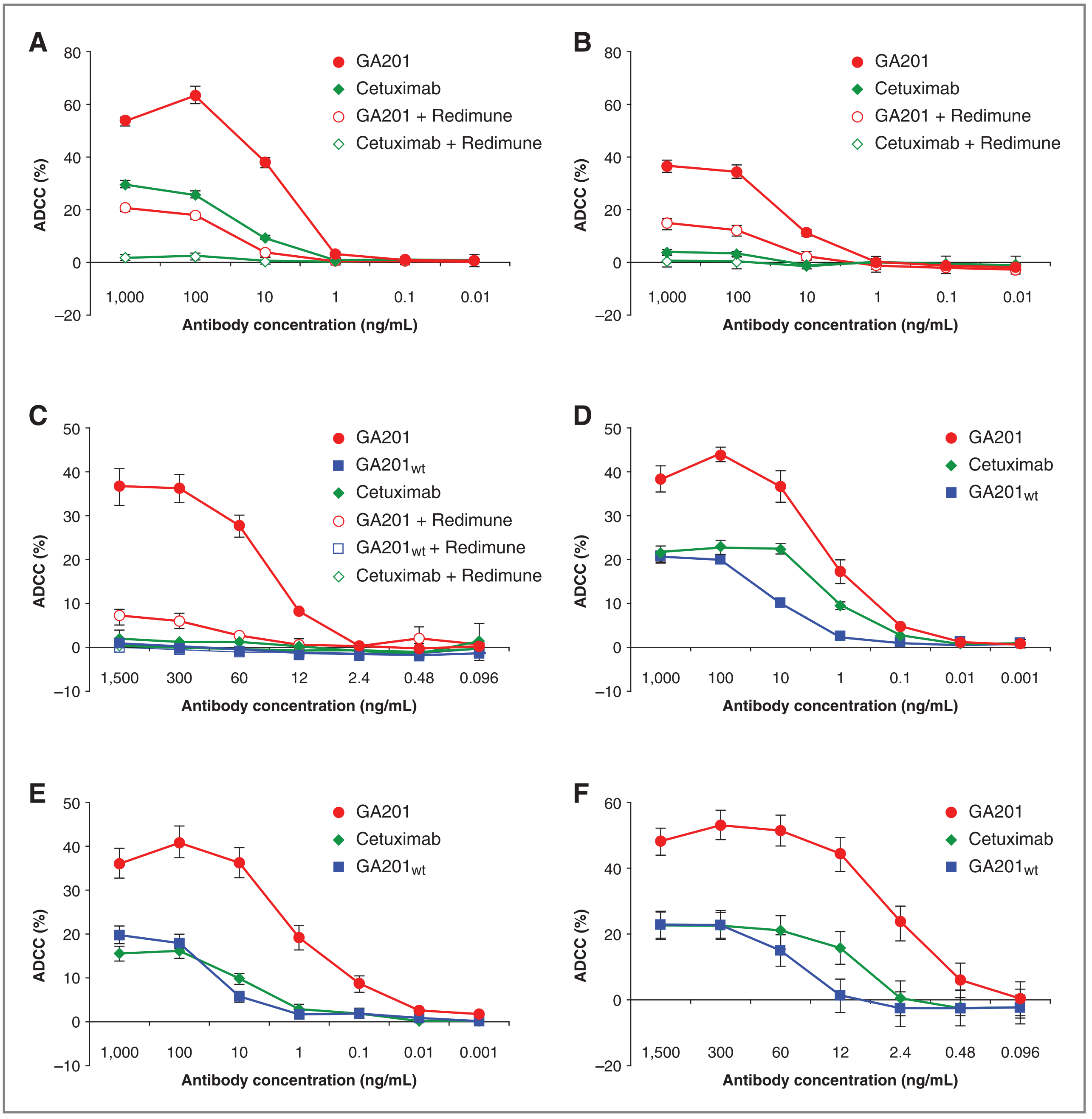

Figure 3. GA201 exhibits superior *in vitro* ADCC activity compared with cetuximab in a variety of scenarios. The figure shows the result of ADCC assays conducted using effector and target cells at a ratio of 3:1 (A–C) or 25:1 (D–E). Cell cytotoxicity was measured by quantifying lactose dehydrogenase activity released from damaged/dying cells. Figures show graphs from representative experiments. A, A431 epidermoid carcinoma (EGFR-overexpressing, *KRAS*-wild-type) cells as target cells and human NK-92 (V158) NK cells (high-affinity FcγRIIIA) as effector cells. B, A431 cells as target cells and human NK-92 (F158) NK cells (low-affinity FcγRIIIA) as effector cells. C, A549 adenocarcinomic human alveolar basal epithelial (low EGFR expression, *KRAS*-mutant) cells as target cells and human NK-92 (V158) NK cells as effector cells. D, MKN45 human gastric adenocarcinoma (*KRAS*-wild-type) cells as target and human PBMCs as effector cells. E, H266 NSCLC cells as target and human PBMCs as effector cells. F, A549 cells as target and human PBMCs as effector cells.

compared with the nonglycoengineered version ($GA201_{wt}$; $P < 0.05$) or vehicle control ($P < 0.005$; Fig. 4B).

GA201 also showed superior efficacy to cetuximab in another lung model. H460M2 cells, an aggressive NSCLC cell line (very low EGFR expression, *KRAS*-mutant), were engrafted into the lung of SCID human FcγRIIIA transgenic mice (bearing murine FcγRIV-positive macrophages and human FcγRIIIA-positive transgenic murine NK cells as

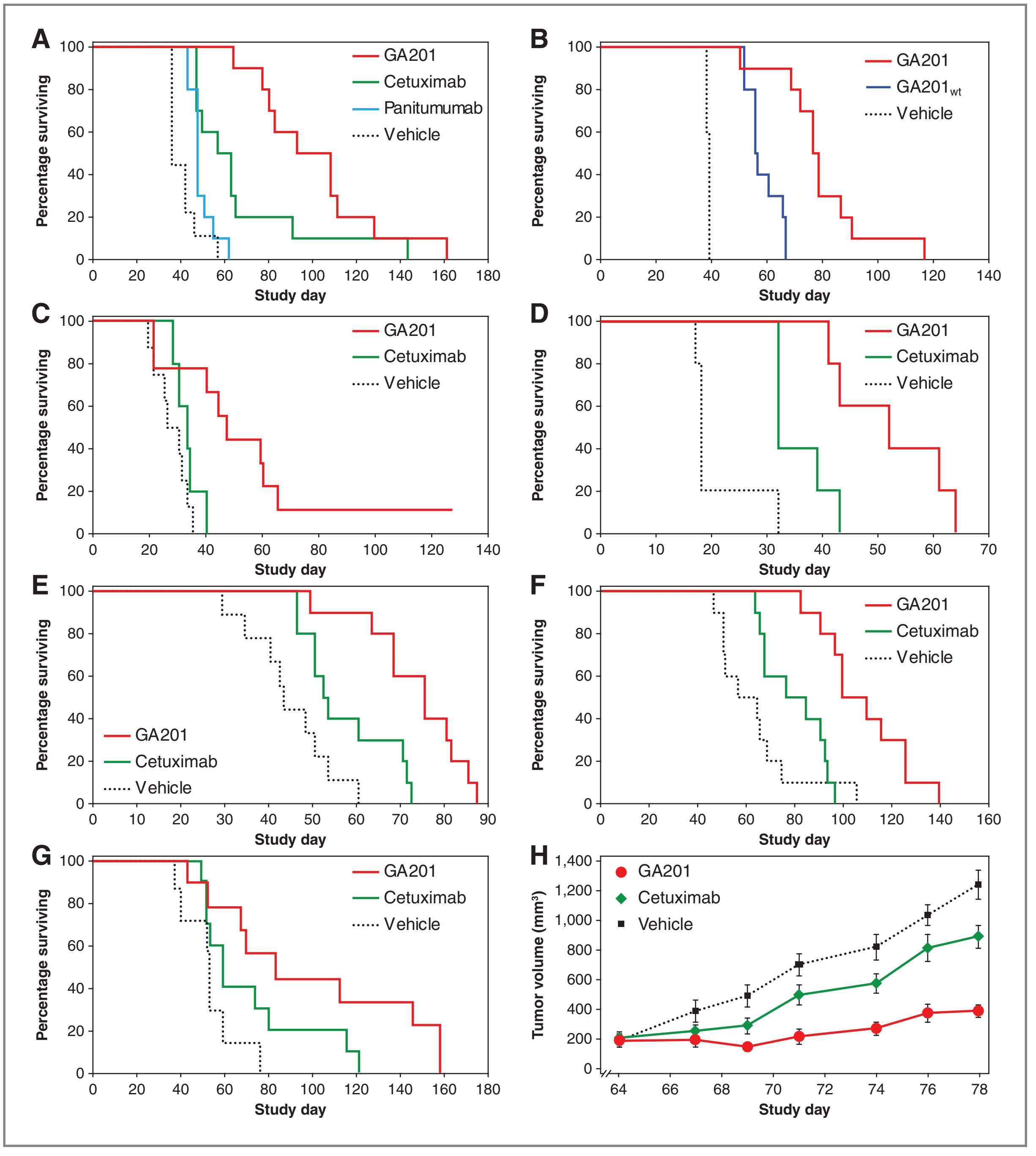

Figure 4. Superior efficacy of GA201 versus cetuximab in xenograft models. Xenograft models were established using either SCID/beige mice (which express active FcγRIV-positive murine macrophages as effector cells; A, B, D, F, G, and H) or human FcγRIIIA transgenic SCID mice (which express both murine FcγRIV-positive macrophages and human FcγRIIIA-positive transgenic murine NK cells as effectors; C and E). All animals ($n = 10$ per treatment group) were treated therapeutically with 25 mg/kg antibody once weekly for 3 weeks or as a single dose (as indicated later). Dosing began on day 7 after injection of tumor cells, once tumor was detectable. A–G, the proportion of study animals surviving according to study day. The termination criterion for sacrificing animals was sickness with locomotion impairment. A, A549 lung adenocarcinoma (low EGFR expression, *KRAS*-mutant) xenograft model. B, The same A549 model treated with a single dose of antibody. C, H460M2 NSCLC (very low EGFR expression, *KRAS*-mutant) xenograft model. D, LS174T (low EGFR expression, *KRAS*-mutant) colorectal xenograft model treated with a single dose of antibody. E, HT29 colorectal (low EGFR expression, *KRAS*-wild-type) xenograft model. F, Panc-1 pancreatic cancer (medium EGFR expression, *KRAS*-mutant) xenograft model. G, ACHN renal cell carcinoma (medium EGFR expression, *KRAS*-mutant) xenograft model. The data show that GA201 achieved a significantly superior median OS compared with cetuximab, panitumumab, nonglycoengineered $GA201_{wt}$, or vehicle control in all orthotopic xenograft models investigated. H, depicts tumor volume according to study day in a MDA-MB-231 mammary cell carcinoma (medium EGFR expression, *KRAS*-mutant) xenograft model. Data are presented as mean and SD and show superior tumor growth inhibition with GA201 compared with cetuximab or vehicle control.

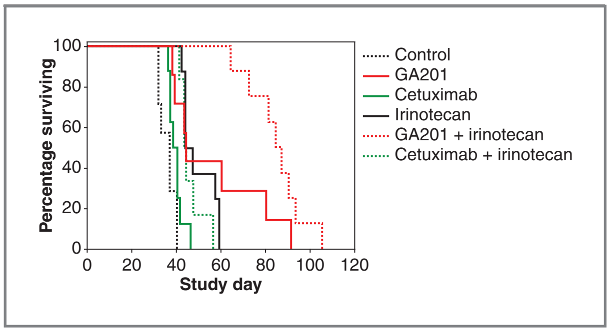

Figure 5. Enhanced efficacy of GA201 in combination with irinotecan in an orthotopic EGFR-positive HT29 tumor model. HT29 CRC cells were established in the human FcγRIIIA transgenic SCID mouse ($n = 10$ per treatment group). Therapy with either saline (control), single-agent antibody (30 mg/kg), irinotecan (30 mg/kg), or the combination of mAb plus irinotecan was initiated on day 7 and given once weekly for 3 weeks.

effectors). In this setting of low EGFR expression and mutant *KRAS*, cetuximab showed no significant difference in survival compared with control ($P = 0.187$), whereas GA201 showed a significant therapeutic benefit ($P = 0.002$; Fig. 4C). Interestingly, when this experiment was done under identical conditions, but implanted in SCID/beige instead of human FcγRIIIA transgenic mice, neither antibody mediated a significant increase in survival (data not shown), indicating the relative importance of FcγRIIIA-dependent ADCC mediated by NK cells in this aggressive tumor model.

To evaluate GA201 efficacy in CRC models, 2 different liver metastatic xenograft models were established. LS174T cells (low EGFR expression, *KRAS*-mutant) were injected intrasplenically into SCID/beige mice and HT29 cells (low EGFR expression, *KRAS*-wild-type) into human FcγRIIIA transgenic SCID mice. As a *KRAS*-wild-type cell line, the HT29 cell line model is sensitive to both of GA201's therapeutic modes of action (MoA; EGFR signaling inhibition and ADCC). In both CRC models, GA201 proved to be superior to cetuximab ($P < 0.05$ for both models) and vehicle control ($P < 0.001$ for both models; Fig. 4D and E).

The superior efficacy of GA201 compared with cetuximab was further shown in a SCID/beige mice pancreatic orthotopic model (Panc-1 cells—medium EGFR expression, *KRAS*-mutant; Fig. 4F) and orthotopic renal cell carcinoma (ACHN cells—medium EGFR expression and *KRAS*-mutant; Fig. 4G).

The ability of GA201 to inhibit tumor growth was further investigated in an orthotopic breast cancer model using the MDA-MB-231 triple-negative (estrogen receptor, progesterone receptor, and HER2 negative) mammary cell line (medium EGFR expression and *KRAS*-mutant). MDA-MB-231 cells were injected into the mammary gland of SCID/beige mice and animals were treated with 25 mg/kg i.v. GA201 or cetuximab every 3 weeks beginning when tumors reached 200 mm^3. GA201 exhibited significantly better antitumoral efficacy compared with both cetuximab ($P = 0.0054$) and vehicle control ($P < 0.0001$; Fig. 4H).

Enhanced efficacy of GA201 in combination with chemotherapy

The efficacy of GA201 was potentiated when combined with chemotherapy in the HT29 CRC human FcγRIIIA transgenic SCID mouse model. Mice were treated with single-agent mAb (30 mg/kg), single agent irinotecan (30 mg/kg), or combination therapy. Median OS was significantly longer with GA201 plus irinotecan compared with single-agent GA201 ($P = 0.013$), cetuximab ($P = 0.0002$), and irinotecan ($P = 0.0002$), or cetuximab plus irinotecan ($P = 0.0002$; Fig. 5).

Immunohistopathologic analysis of xenograft tumors

Immunohistopathologic analysis of tumor tissue from an A549 cell SCID human FcγRIIIA transgenic mouse xenograft model indicated a significant increase in the number of active murine CD68+ macrophages (immune effector cells able to mediate ADCC) in the tumors of animals treated with GA201 compared with animals receiving cetuximab ($P = 0.0055$) or vehicle control ($P = 0.0099$; Fig. 6A–C). Twenty-four hours after therapeutic injection of a single dose (25 mg/kg) of either GA201 or cetuximab, a 4- to 5-fold difference in the number of infiltrating CD68+ cells was observed with GA201-treated animals compared with animals receiving cetuximab or vehicle control. A similar increase in the number of infiltrating NKp46+ cells was seen (Fig. 6E–G).

Discussion

Anti-EGFR antibodies have proven to be valuable tools in the treatment of EGFR-positive cancers; however, these agents are ineffective against patients with CRC with tumors carrying mutations in the *KRAS* oncogene. Furthermore, even in the absence of *KRAS* mutation, only a subset of patients with CRC will benefit from treatment with cetuximab and panitumumab. Response rates to cetuximab in patients with wild-type *KRAS* CRC with tumors carrying *BRAF* or *NRAS* mutations are significantly lower than in patients without mutation in these genes (45). Using a panel of 4 genes (*KRAS*, *BRAF*, *PIK3CA*, and *PTEN*), Sartore-Bianchi and colleagues showed response rates to cetuximab and panitumumab of 51% in patients with CRC with no mutations compared with 4% when 1 gene was mutated and no responses when 2 or more genes were mutated (46). These data indicate that inhibition of ligand binding and EGFR signaling alone is insufficient when the oncogenic stimulus arises downstream of the EGFR. Here, we report the development of GA201, a novel, humanized anti-EGFR antibody with a dual MoA. As well as inhibiting the binding of EGF and EGFR receptor dimerization, GA201 was specifically glycoengineered for enhanced ADCC activity. We investigated the *in vitro* and *in vivo* activity of GA201 and compared it with cetuximab.

GA201 inhibited EGF binding, EGFR receptor dimerization, and downstream signaling with a similar potency to cetuximab; however, GA201 was superior to cetuximab in all cell killing assay systems tested. Glycoengineering significantly increased the affinity of GA201 for both the low-

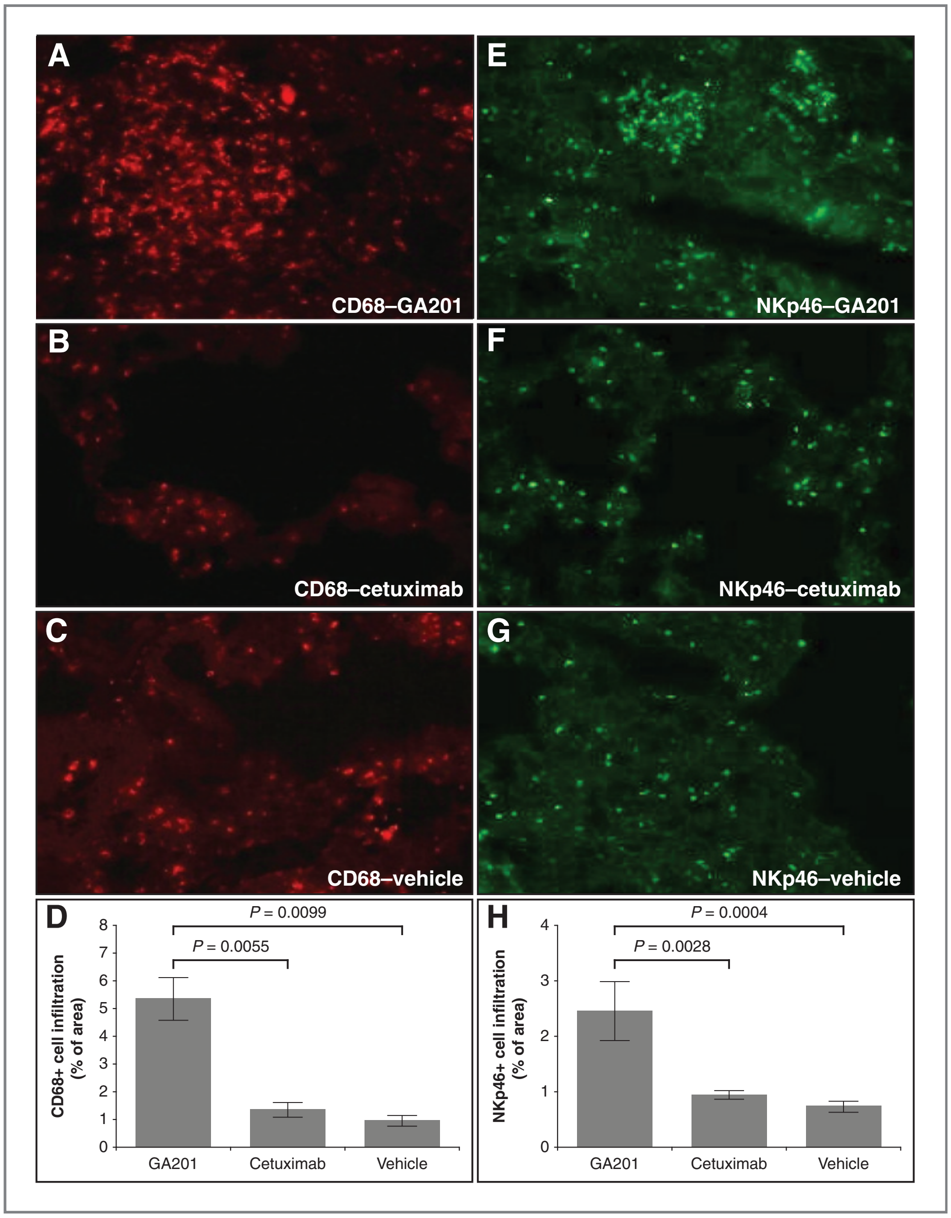

Figure 6. Tumor infiltration of CD68+ and NKp46+ immune cells in response to GA201 treatment in A549 NSCLC tumor xenografts in SCID human FcγRIIIA transgenic mice. A549 NSCLC tumor xenografts in mice bearing both murine FcγRIV-positive murine macrophages and human FcγRIIIA-positive murine NK cells as effectors were treated with a single therapeutic injection of 25 mg/kg GA201 or cetuximab or PBS alone (control). After 24 hours, the lungs were recovered and CD68+ cells (A–C) and NKp46+ cells (E–F) were detected using fluorescent immunohistochemistry on frozen sections. A 4- to 5-fold difference was observed in the number of infiltrating CD68+ immune cells and a 2- to 3-fold difference was seen in the number of infiltrating NKp46+ cells, with GA201 compared with cetuximab and control groups.

and high-affinity human FcγRIIIA variants compared with cetuximab. Importantly, binding to the low-affinity FcγRIIIA variant was substantially increased, such that it exceeded that of cetuximab for the high-affinity variant. This translated into superior efficacy *in vitro* (2- to >5-fold) in ADCC assays compared with cetuximab. Calculating potency based on the concentration of each antibody required to achieve 50% of its respective maximal ADCC resulted in 3- to 10-fold enhancements in potency with GA201, whereas calculating potency based on the concentration of GA201 required to reach the maximal ADCC of cetuximab indicated a more than 100-fold increase in potency versus cetuximab.

The influence of Fc-region glycoengineering on the ADCC activity of GA201 was clearly shown by comparison with the nonglycoengineered, wild-type GA201$_{wt}$. The efficacy of GA201$_{wt}$ was similar to that of cetuximab. In contrast to cetuximab, the ADCC activity of GA201 was retained in the presence of physiologic concentrations of nonspecific total human IgG. The enhanced *in vitro* ADCC activity of GA201 translated into significantly increased survival in a series of orthotopic xenograft models compared with cetuximab. These models showed improved efficacy with GA201 regardless of the tumor type, EGFR expression level, or *KRAS* mutation status. Combining GA201 with irinotecan further improved efficacy over GA201 monotherapy.

Immunohistochemical analysis of tumor biopsies from an A549 cell SCID human FcγRIIIA transgenic mouse xenograft model showed that the activity of GA201 was associated with a significantly greater tumor infiltration of both murine CD68+ macrophage cells and NKp46+ NK cells compared with cetuximab. Immune effector cell recruitment into tumors has been shown with other mAbs. Following trastuzumab-based therapy, an increase in the number of tumor-infiltrating NK cells (but not macrophages) was seen in patients with breast cancer, and this increase was significantly greater compared with patients receiving non-trastuzumab-based therapies increased significantly following trastuzumab treatment (47). Furthermore, patients showing a pathologic response tended to show an increased number of infiltrating NK cells compared with poor or nonresponders. While no difference was seen in the number of infiltrating CD56+ NK in a trial of patients with CRC treated with cetuximab compared with noncetuximab-treated patients, tumor-infiltrating CD56+ cells were an independent predictor of PFS and response only in patients treated with cetuximab-based therapies (48).

The immune cell infiltration seen with GA201, together with the very significant efficacy difference observed between the glycoengineered GA201 and its wild-type non-glycoengineered version, suggest that the *in vivo* efficacy of GA201 in such models is likely attributable to the superior immune effector functions of GA201 resulting from an increased binding affinity to FcγRIV and FcγRIIIA on infiltrating mouse monocytes, macrophages and NK cells.

The majority of cell lines used in both the ADCC assays and xenograft models were mutant for the *KRAS* oncogene and consequently would be expected to be less sensitive to signal inhibition via EGFR receptor blockade. This provides further evidence that the superior efficacy seen in animal models is likely due to the enhanced ADCC properties of GA201, and indicates that GA201 might be more efficacious in patients with *KRAS*-mutated tumors as compared with non-ADCC-enhanced anti-EGFR antibodies. Clinical studies investigating the safety and efficacy of GA201 are ongoing. A recently completed phase I dose-escalation trial of GA201 showed an acceptable safety profile and promising efficacy (49). In a heavily pretreated population of 75 patients with advanced and/or metastatic solid tumors, single-agent GA201 achieved 1 complete response and 2 partial responses in patients with CRC, including a partial response in 1 patient with a *KRAS*-mutant tumor. Further development of GA201 is now ongoing investigating the efficacy of GA201 in a population of patients with *KRAS*-mutant CRC and in combination with standard chemotherapy regimens in CRC and NSCLC.

In conclusion, GA201 showed significantly improved cell killing in *in vitro* ADCC-based assays and preclinical *in vivo* tumor models using both *KRAS*-wild-type and *KRAS*-mutated tumor cells. Given that GA201 shares a similar MoA to cetuximab and panitumumab (such as blocking of ligand binding) but with significantly greater ADCC, GA201 may offer not only improved efficacy over cetuximab and panitumumab in patients with EGFR-positive solid tumors, but may represent a first-in-class treatment of patients with *KRAS*-mutated tumors.

Disclosure of Potential Conflicts of Interest

C.A. Gerdes is Head of Preclinical Oncology, V. Nicolini is Head of Histophatology, S. Herter is Senior Scientist, E. van Puijenbroek is Scientist/Team leader, and S. Lang is Principal Associate in Roche Glycart AG.

Authors' Contributions

Conception and design: C.A. Gerdes, V. Nicolini, E. Moessner, T. Friess, C. H. Ries, B. Bossenmaier, P. Umaña
Development of methodology: C.A. Gerdes, V. Nicolini
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.A. Gerdes, V. Nicolini, M. Roemmele, O. Freytag, T. Friess, C.H. Ries
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.A. Gerdes, V. Nicolini, T. Friess, C.H. Ries
Writing, review, and/or revision of the manuscript: C.A. Gerdes, V. Nicolini, O. Freytag, T. Friess, C.H. Ries, B. Bossenmaier, H.J. Mueller, P. Umaña
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.A. Gerdes, S. Herter, E. van Puijenbroek, S. Lang, M. Roemmele
Study supervision: C.A. Gerdes, T. Friess, P. Umaña

Acknowledgments

The authors thank Priska Rueegg, Daniela Ackermann, Dominique Bandi, and Michael Mauch for quality administrative support and the functional support of Roche Glycart molecular biology and process biochemistry. The authors also thank Jon Chick, Luigi Manenti, Joanne Barrett, and the rest of the Roche GA201 global team for discussion contributions while generating this work.

Support for third-party writing assistance for this article, furnished by Jamie Ashman, was provided by Prism Ideas.

Grant Support

This study and editorial support for the preparation of the article were funded by Roche Glycart AG, Switzerland.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 26, 2012; revised October 5, 2012; accepted November 4, 2012; published OnlineFirst December 3, 2012.

References

1. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995;19:183–232.
2. Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. Eur J Cancer 2001;37(Suppl 4):S9–15.
3. Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. Oncogene 2000;19:6550–65.
4. Wakeling AE, Guy SP, Woodburn JR, Ashton SE, Curry BJ, Barker AJ, et al. ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. Cancer Res 2002;62: 5749–54.
5. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
6. Jackman DM, Yeap BY, Sequist LV, Lindeman N, Holmes AJ, Joshi VA, et al. Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non–small cell lung cancer patients treated with gefitinib or erlotinib. Clin Cancer Res 2006;12: 3908–14.
7. Van Cutsem E, Peeters M, Siena S, Humblet Y, Hendlisz A, Neyns B, et al. Open-label phase III trial of panitumumab plus best supportive

care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. J Clin Oncol 2007;25:1658–64.

8. Van Cutsem E, Kohne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med 2009;360:1408–17.
9. Sobrero AF, Maurel J, Fehrenbacher L, Scheithauer W, Abubakr YA, Lutz MP, et al. EPIC: phase III trial of cetuximab plus irinotecan after fluoropyrimidine and oxaliplatin failure in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:2311–9.
10. Andreyev HJ, Norman AR, Cunningham D, Oates JR, Clarke PA. Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. J Natl Cancer Inst 1998;90:675–84.
11. Amado RG, Wolf M, Peeters M, Van CE, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:1626–34.
12. Van Cutsem E, Kohne CH, Lang I, Folprecht G, Nowacki MP, Cascinu S, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. J Clin Oncol 2011;29:2011–9.
13. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 2006;66:3992–5.
14. Dempke WC, Heinemann V. Ras mutational status is a biomarker for resistance to EGFR inhibitors in colorectal carcinoma. Anticancer Res 2010;30:4673–7.
15. Green MC, Murray JL, Hortobagyi GN. Monoclonal antibody therapy for solid tumors. Cancer Treat Rev 2000;26:269–86.
16. Ludwig DL, Pereira DS, Zhu Z, Hicklin DJ, Bohlen P. Monoclonal antibody therapeutics and apoptosis. Oncogene 2003;22:9097–106.
17. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. Nat Med 2000;6:443–6.
18. Schneider-Merck T, Lammerts van Bueren JJ, Berger S, Rossen K, van Berkel PH, Derer S, et al. Human IgG2 antibodies against epidermal growth factor receptor effectively trigger antibody-dependent cellular cytotoxicity but, in contrast to IgG1, only by cells of myeloid lineage. J Immunol 2010;184:512–20.
19. Kurai J, Chikumi H, Hashimoto K, Yamaguchi K, Yamasaki A, Sako T, et al. Antibody-dependent cellular cytotoxicity mediated by cetuximab against lung cancer cell lines. Clin Cancer Res 2007;13:1552–61.
20. Pander J, Heusinkveld M, Van der ST, Jordanova ES, Baak-Pablo R, Gelderblom H, et al. Activation of tumor-promoting type 2 macrophages by EGFR-targeting antibody cetuximab. Clin Cancer Res 2011;17:5668–73.
21. Patel D, Guo X, Ng S, Melchior M, Balderes P, Burtrum D, et al. IgG isotype, glycosylation, and EGFR expression determine the induction of antibody-dependent cellular cytotoxicity *in vitro* by cetuximab. Hum Antibodies 2010;19:89–99.
22. Jefferis R. Antibody therapeutics: isotype and glycoform selection. Expert Opin Biol Ther 2007;7:1401–13.
23. Bibeau F, Lopez-Crapez E, Di FF, Thezenas S, Ychou M, Blanchard F, et al. Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 2009;27:1122–9.
24. Steplewski Z, Sun LK, Shearman CW, Ghrayeb J, Daddona P, Koprowski H. Biological activity of human-mouse IgG1, IgG2, IgG3, and IgG4 chimeric monoclonal antibodies with antitumor specificity. Proc Natl Acad Sci U S A 1988;85:4852–6.
25. Nimmerjahn F, Ravetch JV. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science 2005;310: 1510–2.
26. Schlaeth M, Berger S, Derer S, Klausz K, Lohse S, Dechant M, et al. Fc-engineered EGF-R antibodies mediate improved antibody-dependent cellular cytotoxicity (ADCC) against KRAS-mutated tumor cells. Cancer Sci 2010;101:1080–8.
27. Niwa R, Sakurada M, Kobayashi Y, Uehara A, Matsushima K, Ueda R, et al. Enhanced natural killer cell binding and activation by low-fucose IgG1 antibody results in potent antibody-dependent cellular cytotoxicity induction at lower antigen density. Clin Cancer Res 2005; 11:2327–36.
28. Mossner E, Brunker P, Moser S, Puntener U, Schmidt C, Herter S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. Blood 2010;115: 4393–402.
29. Niwa R, Shoji-Hosaka E, Sakurada M, Shinkawa T, Uchida K, Nakamura K, et al. Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. Cancer Res 2004;64:2127–33.
30. Ravetch JV, Perussia B. Alternative membrane forms of Fc gamma RIII (CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. J Exp Med 1989;170:481–97.
31. Zhang W, Gordon M, Schultheis AM, Yang DY, Nagashima F, Azuma M, et al. FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. J Clin Oncol 2007;25:3712–8.
32. Pander J, Gelderblom H, Antonini NF, Tol J, van Krieken JH, Van der ST, et al. Correlation of FCGR3A and EGFR germline polymorphisms with the efficacy of cetuximab in KRAS wild-type metastatic colorectal cancer. Eur J Cancer 2010;46:1829–34.
33. Musolino A, Naldi N, Bortesi B, Pezzuolo D, Capelletti M, Missale G, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. J Clin Oncol 2008;26: 1789–96.
34. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. J Clin Oncol 2003;21:3940–7.
35. Queen C, Schneider WP, Selick HE, Payne PW, Landolfi NF, Duncan JF, et al. A humanized antibody that binds to the interleukin 2 receptor. Proc Natl Acad Sci U S A 1989;86:10029–33.
36. Ferrara C, Brunker P, Suter T, Moser S, Puntener U, Umana P. Modulation of therapeutic antibody effector functions by glycosylation engineering: influence of Golgi enzyme localization domain and co-expression of heterologous beta1, 4-N-acetylglucosaminyltransferase III and Golgi alpha-mannosidase II. Biotechnol Bioeng 2006;93: 851–61.
37. Brunker P, Sondermann P, Umana P. Glycoengineered therapeutic antibodies. In:Little M, editor. Recombinant antibodies for immunotherapy.New York: Cambridge Univeristy Press; 2009. p. 144–56.
38. Modjtahedi H, Eccles S, Box G, Styles J, Dean C. Immunotherapy of human tumour xenografts overexpressing the EGF receptor with rat antibodies that block growth factor-receptor interaction. Br J Cancer 1993;67:254–61.
39. Modjtahedi H, Moscatello DK, Box G, Green M, Shotton C, Lamb DJ, et al. Targeting of cells expressing wild-type EGFR and type-III mutant EGFR (EGFRvIII) by anti-EGFR MAb ICR62: a two-pronged attack for tumour therapy. Int J Cancer 2003;105:273–80.
40. Dawson JP, Berger MB, Lin CC, Schlessinger J, Lemmon MA, Ferguson KM. Epidermal growth factor receptor dimerization and activation require ligand-induced conformational changes in the dimer interface. Mol Cell Biol 2005;25:7734–42.
41. Brennan PJ, Kumagai T, Berezov A, Murali R, Greene MI. HER2/neu: mechanisms of dimerization/oligomerization. Oncogene 2000;19: 6093–101.
42. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139: 271–9.
43. Ferrara C, Stuart F, Sondermann P, Brunker P, Umana P. The carbohydrate at FcgammaRIIIa Asn-162. An element required for high affinity binding to non-fucosylated IgG glycoforms. J Biol Chem 2006;281: 5032–6.

44. Junttila TT, Parsons K, Olsson C, Lu Y, Xin Y, Theriault J, et al. Superior *in vivo* efficacy of afucosylated trastuzumab in the treatment of HER2-amplified breast cancer. Cancer Res 2010;70:4481–9.
45. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 2010;11:753–62.
46. Sartore-Bianchi A, Di Nicolantonio F, Nichelatti M, Molinari F, De Dosso S, Saletti P, et al. Multi-determinants analysis of molecular alterations for predicting clinical benefit to EGFR-targeted monoclonal antibodies in colorectal cancer. PLoS ONE 2009;4: e7287.
47. Arnould L, Gelly M, Penault-Llorca F, Benoit L, Bonnetain F, Migeon C, et al. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? Br J Cancer 2006;94:259–67.
48. Markasz L, Stuber G, Vanherberghen B, Flaberg E, Olah E, Carbone E, et al. Effect of frequently used chemotherapeutic drugs on the cytotoxic activity of human natural killer cells. Mol Cancer Ther 2007;6: 644–54.
49. Paz Ares L, Gomez Roca C, Delord JP, Cervantes A, Markman B, Corral J, et al. Phase I pharmacokinetic and pharmacodynamic dose-escalation study of RG7160 (GA201), the first glycoengineered monoclonal antibody against the epidermal growth factor receptor (EGFR) in patients with advanced solid tumors. J Clin Oncol 2011;29:3783–90.