RG7116, a therapeutic antibody that binds the inactive HER3 receptor and is optimized for immune effector activation

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Abstract

The epidermal growth factor receptor HER3 is emerging as an attractive cancer therapeutic target due to its central position in the HER receptor signaling network. HER3 amplifies PI3K-driven tumorigenesis and its upregulation in response to other anti-HER therapies has been implicated in resistance to them. Here we report the development and characterization of RG7116, a novel anti-HER3 monoclonal antibody designed to block HER3 activation, downregulate HER3, and mediate enhanced antibody-dependent cellular cytotoxicity (ADCC) via glycoengineering of the Fc moiety. Biochemical studies and X-ray crystallography revealed that RG7116 bound potently and selectively to domain 1 of human HER3. Heregulin binding was prevented by RG7116 at concentrations >1 nM as was nearly complete inhibition of HER3 heterodimerization and phosphorylation, thereby preventing downstream AKT phosphorylation. In vivo RG7116 treatment inhibited xenograft tumor growth up to 90% relative to controls in a manner accompanied by downregulation of cell surface HER3. RG7116 efficacy was further enhanced in combination with anti-EGFR (RG7160) or anti-HER2 (pertuzumab) monoclonal antibodies. Further, the ADCC potency of RG7116 was enhanced compared with the non-glycoengineered parental antibody, both in vitro and in orthotopic tumor xenograft models, where an increased median survival was documented. ADCC degree achieved in vitro correlated with HER3 expression levels on tumor cells. In summary, the combination of strong signaling inhibition and enhanced ADCC capability rendered RG7116 a highly potent HER3 targeting agent suitable for clinical development.

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Introduction

HER3 (ERBB3) is a member of the human epidermal growth factor receptor (HER) family of transmembrane receptors. By homo- and heterodimerization, these receptors form a complex overlapping signaling network that regulates cell differentiation, migration, proliferation, and survival (1;2). HER monomers are predominantly in an inactive “closed” conformation. Activation occurs following ligand binding by receptor dimerization and subsequent transphosphorylation within the dimerized receptor pair. HER3 is unique in that it lacks significant kinase activity (3) and does not form stable homodimers (4); thus it depends on HER1 (EGFR), HER2 (ERBB2), or HER4 (ERBB4) as heterodimerization partners. Within the HER family, HER3 is the most potent in activating the phosphatidylinositol 3-kinase (PI3K/AKT) pathway, since its intracellular domain contains multiple tyrosine phosphorylation sites that recruit the regulatory p85 subunit of PI3K. HER2-HER3 heterodimers constitute a high-affinity co-receptor pair for heregulin (HRG [NRG1]), the predominant ligand for HER3 (5-7), and form the most potent dimeric HER signaling complex – strongly activating diverse cellular pathways such as the RAS/RAF/mitogen-activated protein kinase (MAPK) and the PI3K/AKT pathways (8;9).

Deregulation of HER signaling is a key mechanism by which tumor cells can evade normal growth constraints. Several anti-HER agents are widely used clinically, including the anti-EGFR monoclonal antibody (mAb) cetuximab, the anti-HER2 mAb trastuzumab and the EGFR tyrosine kinase inhibitors erlotinib and gefitinib. The importance of HER3 in cancer has only recently emerged; upregulation of HER3 is an adverse prognostic factor in many tumor types and is associated with worse survival (10-14;14).

HER3 has also been implicated in the development of resistance to anti-EGFR or anti-HER2 therapy (15-17). A shift from HER1 homodimer to HER1/HER3 heterodimer signaling in response to sustained treatment with EGFR inhibitors leads to reactivation of the PI3K/AKT pathway and bypasses the therapeutic action of these compounds (18). Upregulation of
HER3 appears to be driven by negative feedback from AKT (18). Amplification of the tyrosine kinase receptor MET induces HER3 phosphorylation and PI3K activation, representing another mechanism by which HER3 confers resistance to EGFR targeting therapies (19).

Therefore, anti-HER3 mAbs are attractive both as therapeutic inhibitors of HER3 signaling in their own right and as potentiators of other anti-HER therapies. Understanding the molecular basis of signaling inhibition by therapeutic mAbs is important for their clinical development and is greatly helped by combining crystallography, biophysical characterization of antigen/antibody complexes, and thermodynamic analysis of the molecular forces that drive complex formation (20). While the anti-EGFR mAb matuzumab (21) is believed to act solely by blocking conformational changes required for EGF binding and receptor dimerization, the mAbs cetuximab (22), IMC-11F8 (23), and zalutumumab (24) also occlude the ligand-binding site. To achieve highly potent and specific receptor inhibition an enthalpy-driven key-and-lock binding mechanism typical for a fully matured antibody-antigen interaction is advantageous, since it can freeze an inactive receptor conformation by a network of directed interactions at the binding interface.

In addition to signaling inhibition, mAbs of the IgG1 subtype can also exert a therapeutic effect by recruiting host immune effector cells such as natural killer (NK) cells that are capable of killing tumor cells via antibody-dependent cellular cytotoxicity (ADCC). A critical step in the activation of cytotoxic cells is the binding of mAbs to FcyRIIIa (FCGR3A) on immune effector cells, and the strength of this interaction is determined by antibody isotype, the glycosylation pattern of the antibody Fc region and FcyRIIIa polymorphisms (25-27). In particular, the affinity of human IgG1 to FcyRIIIa is higher when oligosaccharides lack the variable fucose attachment (28). Glycoengineering of the Fc portion is an established method for increasing the affinity of therapeutic mAbs for Fcy receptors on human immune effector cells and for enhancing ADCC (28;29).
Our aim was to generate a glycoengineered anti-HER3 IgG1 antibody that would combine complete HER3 signaling inhibition with enhanced ADCC. Here we report the full characterization of this novel therapeutic mAb.
Methods

Cell lines

Human breast (MCF7, MDA-MB-175, T-47D, BT-474, SK-BR3), head and neck squamous (SCCHN; FaDu), pancreatic (BxPC3), and lung (QG56, A549, NCI-H322M, NCI-H1975, HCC827, NCI-H441) cancer cell lines were obtained from the American Type Culture Collection or Immuno-Biological Laboratories (Japan). Cell lines obtained from these suppliers are routinely authenticated by karyotyping, short tandem repeat profiling, assessment of cell morphology, and species verification by isoenzymology. Cell lines were expanded upon receipt and aliquots frozen. Cells were not passaged for more than 6 months after resuscitation. Cells were cultured in RPMI/1640 or MEM Eagle medium (PAN Biotech GmbH) containing 10% fetal calf serum (FCS; PAA Laboratories) at 37°C in a water-saturated atmosphere containing 5% CO2. Using standard recombinant techniques, a series of A549 transfectant cell clones were generated that overexpressed HER3 at different levels (measured by flow cytometry).

Production and glycoengineering of RG7116

NMRI mice were immunized with recombinant HER3 extracellular domain (ECD). The antibody described here (RG7116) was selected from multiple anti-HER3 clones examined based on the results described below. Humanization was performed as described previously (30) and glycoengineering of RG7116 was achieved using GlycoMaB® technology (Roche Glycart AG) (31;32). Glycoengineered RG7116 exhibited a 50-fold higher binding affinity for FcγRIIIa than non-glycoengineered RG7116.

Surface plasmon resonance

The equilibrium and transition state thermodynamic profiles of cetuximab and RG7116 were determined by surface plasmon resonance technology. Temperature-dependent kinetics were measured using a T200 instrument (GE Healthcare, Biacore). A CM5 series S sensor
was used according to the manufacturers’ instructions (system buffer: 10 mM HEPES pH 7.4, 150 mM NaCl, 0.05% TWEEN 20; sample buffer: system buffer supplemented with 1 mg/mL carboxymethyl-dextran; Sigma). An anti-human antibody capture system was established on the biosensor and 8,000 relative response units of goat-anti-human Fcγ fragment-specific polyclonal antibody (Jackson Laboratories) was immobilized using EDC/NHS chemistry. The sensor was deactivated using 1 M ethanolamine.

The temperature-dependent binding kinetics (33) of cetuximab and RG7116 were measured between 13–37°C. Cetuximab was captured by a 1 minute injection (10 µL/min) of 10 nM (13°C) 8 nM (17°C), 7 nM (21°C), 6 nM (25–34°C), 5 nM (37°C). Similarly, RG7116 was captured at concentrations of 7 nM (13°C), 6 nM (17°C) and 5 nM (21–37°C). HER1-ECD (68 kDa) and HER3-ECD (68 kDa) were injected (100 µL/min) in a concentration series of 90 nM, 30 nM, 2 x10 nM, 3.3 nM, 1.1 nM, and 0 nM (buffer control). The association phase was monitored for 3 minutes and the dissociation phase measured for 15 minutes. Acidic sensor regeneration was done using three consecutive injections of 10 mM glycine pH 1.5 (30 µL/min) for 30 seconds. Kinetic parameters of the antibody/receptor domain were calculated (Supplementary Material).

**Crystallization, structure determination and refinement**

The HER3-ECD::Fab(RG7116) complex was crystallized at 20°C using hanging drop vapor diffusion. HER3-ECD::Fab(RG7116) crystallized in space group P1 with one molecule per asymmetric unit. Data to a limiting resolution of 2.64 Å was collected at the European Synchrotron Radiation Facility (Beamline ID23-2, France). The HER3-ECD::Fab(RG7116) structure was solved by molecular replacement using Phaser (34). The apo-structure of human HER3-ECD (PDB accession code 1M6B) (35), a homology model of the variable region of the Fab(RG7116) fragment, and a previously reported structure of the Fab constant region (PDB accession code 3U1S) (36) were used as search models. Protein preparation,
crystallization, structure determination and refinement statistics, including a crystallographic table, are described in the Supplemental Material.

**Heregulin (HRG) displacement assay**

RG7116 diluted in Delfia® Binding Buffer (PerkinElmer) was added to streptavidin binding protein-tagged HER3-ECD immobilized onto streptavidin-coated microtitre plates (final concentration 10^{-3} to 10^{3} nM). Plates were incubated at room temperature for 2 hours with 10 nM europium-labeled HRG-beta (PeproTech) with agitation. After three washes with Delfia Wash Buffer, plates were incubated in Delfia Enhancement Solution for 15 minutes and scanned on a Tecan Infinite F200 reader using a time-resolved fluorescence measurement protocol.

**Inhibition of HER3 and AKT phosphorylation by RG7116**

Serial RG7116 concentrations were incubated with MCF7 or FaDu cells for 50 minutes at 37ºC/5% CO₂. HRG was added (final concentration 500 ng/mL) and cells incubated for another 10 minutes. For BT-474, SK-BR3 and MDA-MB-175 cells, RG7116 was incubated without HRG stimulation. Following PBS washes, cells were lysed in Triton Lysis Buffer (1% Triton-X-100; 10 µg/mL aprotinin; 0.4 mM orthovanadate; 1 mM phenylmethylsulfonyl fluoride) and lysates were denatured in NuPAGE Sample Reducing Agent at 70ºC for 10 minutes. Standard SDS-PAGE and Western blotting was performed using an anti-phospho-HER3 (clone 21D3 [Tyr^{1289}]; Cell Signaling Technologies) or anti-HER3 (clone C-17; Santa Cruz) antibody. Signal was detected using electrochemiluminescence (Amersham) and percent inhibition of HER3 receptor phosphorylation calculated. For analysis of phospho-HER3 in BxPC3 xenograft tumors: lysates were prepared, 20 µg protein/lane was separated by SDS-PAGE, and Western blotting for HER3 and phospho-HER3 was performed.

Inhibition of AKT phosphorylation was examined in MCF7 cells treated with different concentrations of RG7116 and 5 ng/L HRG. Following cell lysis, phosphorylated AKT levels
were measured using the anti-phospho-AKT (pSer<sup>473</sup>) enzyme immune assay kit (Enzo Life Sciences).

**In vitro inhibition of proliferation of MDA-MB-175 cells**

20,000 cells/well were seeded into microtitre plates with DMEM/F12 medium containing 10% FCS and incubated at 37°C/5% CO<sub>2</sub> for one day. Serial RG7116 dilutions were added and further incubated for six days. Cell viability was assessed using the alamarBlue® assay (Invitrogen). EC<sub>50</sub> values were calculated using means of triplicates for each antibody concentration.

**Immunohistochemistry**

Immunohistochemistry of formalin-fixed, paraffin-embedded BxPC3 xenografts was performed semi-automatically on a DAKO autostainer using the DAK-H3-IC anti-HER3 mouse antibody (Dako).

**FACS**

RG7116 (10 μg/mL) was added to T-47D cells and membrane HER3 levels were measured by FACS. A phycoetherin-labeled secondary mAb was used to detect levels of RG7116 bound to surface HER3. 10000 cells were measured and the geometric means of the fluorescence signal were normalized to untreated cells (representing background fluorescence levels).

**Mouse xenograft models**

Subcutaneous xenograft models were generated using human tumor cell lines or by implantation of human tumor fragments. Cell lines and fragments were selected based on a high phospho-HER3/HER3 ratio (analyzed by Western blot). All experiments were conducted according to German Animal Welfare Act guidelines.
5–10 x10^6 cells were injected subcutaneously (s.c.) into female SCID-beige (BxPC3, QG56, A549, NCI-H322M, NCI-H441, FaDu) or Balb/c nude mice (HCC827, NCI-H1975) (Charles River). Mice (n=10 per group) were randomized on Day 21–24 (stratified for primary tumor size) and treatment started. RG7116 (10–25 mg/kg, n=2–5 doses) was given once weekly intraperitoneally (i.p.), RG7160 (anti-EGFR) at 25 mg/kg once weekly i.p., and pertuzumab (anti-HER2) at 15 mg/kg once weekly i.p. with a 2-fold loading dose first. The percentage tumor growth inhibition (TGI) compared with controls (saline treated) was calculated (Supplemental Material).

Subcutaneous patient-derived tumor xenografts models were evaluated at Oncotest GmbH (Freiburg, Germany) or EPO GmbH (Berlin, Germany) by transplantation of small human tumor fragments onto NMRI nude mice. Mice were randomized (n=10 per group) and therapy performed as above.

For an orthotopic cell line-based xenograft mouse model, HER3 recombinant A549-B34 cells were injected i.v. (3x10^6 cells) into female SCID-beige mice (Taconics). Mice (n=15 per group) were randomized when lung tumor growth was confirmed in scout animals (Day 23). Mice received 10–13 weekly i.p. injections of 25 mg/kg RG7116, non-glycoengineered RG7116 or saline control. Median survival was defined as the day when ≥50% of animals/group were sacrificed (termination criterion: body weight loss). Survival data were represented using Kaplan-Meier curves and differences in median survival were compared with the Pairwise Log-Rank test.

**Antibody-dependent cellular cytotoxicity (ADCC) assays**

ADCC was performed using DELFIA BATDA Labeling Reagent and DELFIA Eu-Solution (PerkinElmer) and high-affinity (V158)-FcyRIIa receptor-expressing NK92 effector cells. Effector:target (E:T) ratios of 5:1 (T-47D target cells) or 20:1 (A549 target cells) and a 2-hour incubation time were used. Percent specific cell lysis [(specific release) – (spontaneous release target cell)/(maximum release target cell) – (spontaneous release target cell)] was
calculated from the mean of triplicates and plotted against antibody concentration in ng/mL using a 4-parameter fit.
Results

Affinity of RG7116 for human HER3

RG7116 bound selectively and with high affinity to the ECD of human HER3. Using surface plasmon resonance, the binding affinity of RG7116 to immobilized HER3-ECD was compared with that of cetuximab to HER1.

Kinetic Data

The affinities (KD values) at 25°C were 0.5 nM for RG7116 and 1.7 nM for cetuximab. The 3-fold higher affinity of RG7116 is due to a much slower dissociation rate compared with cetuximab: the association (kₐ) and dissociation (k₆) rate constants for the RG7116/HER3 interaction were 1.79x10⁵ 1/Ms and 8.82x10⁻⁵ 1/s, respectively, and for the cetuximab/HER1 interaction were 6.78x10⁵ 1/Ms and 1.12x10⁻³ 1/s, respectively. RG7116 showed a higher antigen complex stability than cetuximab (t₁/₂-diss = 131 minutes vs 10 minutes for cetuximab), a highly desirable feature for immune effector cell recruitment. Kinetic data for the complete temperature gradient are presented in Supplementary Table S1. At RMAX, both antibodies bound their respective antigen in solution with a molar ratio of 2.

Thermodynamic data

A comprehensive thermodynamic analysis of the conformational states in the antibody/receptor interactions was performed and the energy transition of the free standard binding enthalpy (ΔG°‡), standard binding enthalpy (ΔH°‡) and standard binding entropy (TΔS°‡) are shown in Figure 1, with detailed data in Supplementary Table S2 and Figure S1.

Structure of the HER3-ECD::Fab(RG7116) complex

To investigate the molecular mechanism underlying the very potent inhibition of HER3 signaling by RG7116, we determined the crystal structure of a HER3-ECD::Fab(RG7116) complex with a maximal resolution of 2.6 Å by molecular replacement. In agreement with previous epitope mapping results, the complex structure reveals a specific interaction of
Fab(RG7116) with domain 1 of HER3-ECD (Figure 2A). HER3-ECD is a monomer within the complex with a conformation highly similar to the previously reported apo-HER3-ECD structure (Supplementary Figure S2A) (35). This conformation is commonly accepted to represent the ligand unbound, “inactive” state of the receptor, in which an intramolecular tethering interaction between β-hairpin loops from domain 2 and 4 prevents receptor dimerization (Figure 2A) (37).

The accuracy of the structure was verified by calculating a 2Fo-Fc electron density composite omit map with the program CNS (38). Importantly, all residues in the HER3-ECD::Fab(RG7116) interface, including the side chains, are well defined in this map (Supplementary Figure S2B).

Fab(RG7116) forms a broad network of interactions with the L domain-like β-barrel of HER3-ECD domain 1 (Figure 2B-C). The calculated interface between HER3 and Fab(RG7116) buries an area of 928 Å² (Figure 2B) (39). Both the Fab(RG7116) heavy (608 Å² interface with HER3-ECD) and light chain (373 Å² interface with HER3-ECD) contribute to binding of the HER3-ECD epitope. This corresponds well to other comparable Fab-antigen complexes like cetuximab::EGFR which exhibits a buried surface area of 875 Å² (577 Å² heavy chain::receptor and 358 Å² light chain::receptor interface, respectively) (22).

Epitope binding on HER3-ECD domain 1 is mediated by residues from Fab(RG7116) heavy chain CDR1, CDR2, CDR3 and light chain CDR1 and CDR3 (Figure 2C). The observed interface consists not only of hydrophobic and aromatic-aromatic side chain interactions, but notably of several hydrogen bond, ionic- and cation-pi interactions. Overall, it is of relatively polar nature, which becomes apparent from the electrostatic surface potential map of the Fab(RG7116) epitope (Supplementary Figure S2C).

Binding of Fab(RG7116) leads to almost no significant conformational changes in HER3-ECD domain 1 as a superposition of apo-HER3-ECD (35) and HER3-ECD::Fab(RG7116) complex crystal structures with PyMOL shows a low root mean square deviation of 0.53 Å.
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(Cα atoms only; Supplementary Figure S2D). This is in line with the thermodynamic profile of HER3-ECD::Fab(RG7116) complex formation, which is characteristic for an enthalpy-dominated key-and-lock mechanism (Figure 1). The crystal structure also provides an explanation for the negative entropic term of the HER3-ECD::Fab(RG7116) complex observed in the thermodynamic analysis, as it shows several ordered water molecules in the interface (Figure 2C).

Taken together, the anti-HER3 antibody binds its epitope on the HER3-ECD domain 1 with a well-balanced composition of polar and-non polar interactions “freezing” the inactive, tethered conformation of HER3.

**RG7116 prevents HRG ligand binding and inhibits downstream phosphorylation and cell proliferation**

The ability of RG7116 to competitively inhibit the binding of HRG to HER3 and prevent receptor phosphorylation, downstream signaling and cell proliferation was investigated *in vitro*. In a Delfia assay, RG7116 prevented binding of HRG to the HER3-ECD with an IC$_{50}$ of 0.13 nM (Figure 3A). Bound HRG was completely displaced at RG7116 concentrations ≥1 nM. This translated into a dose-dependent inhibition of HER3 phosphorylation in MCF7 (breast) and FaDu (hypopharynx) cancer cells. HER3 phosphorylation in MCF7 cells induced by 70 nM HRG was almost completely inhibited (>85%) when co-incubated with RG7116 at concentrations >0.3 µg/mL (2 nM; Figure 3B). A similar complete inhibition of HER3 phosphorylation was seen in FaDu cells with 0.1 µg/mL (0.7 nM) RG7116. Furthermore, RG7116 inhibited downstream AKT phosphorylation in MCF7 cells with an IC$_{50}$ of 0.42 nM (0.06 µg/mL; Figure 3C).

HER3 activation in the presence of ligand and HER3 activation by overexpressed HER2 may not follow the same biological mechanism; therefore, the effect of RG7116 on phospho-HER3 levels in the absence of extraneous added HRG ligand was measured. Inhibition of phospho-HER3 levels was seen using a series of breast cell lines (Figure 3D), including the
MDA-MB-175 cell line in which the oncogenic signal arises from an autocrine HRG growth loop. RG7116-induced downregulation of membrane HER3 in the absence of HRG was investigated in T-47D breast cells. A time-dependent decrease in surface HER3 was demonstrated by FACS (Figure 3E), reaching levels seen in unstained control cells by 5 hours post-RG7116 treatment.

The anti-proliferative potential associated with blocking HER3 signaling by RG7116 was examined in vitro using MDA-MB-175 human breast cancer cells (doubling time ~1.5 days). After 6 days incubation with a range of RG7116 concentrations, the maximal growth inhibition achieved was approximately 60% compared with control cell (Figure 3F). The EC50 for RG7116-mediated growth inhibition was 8.5 nM.

**RG7116 treatment results in strong TGI of mouse xenograft tumors**

The in vivo activity of RG7116 was investigated using subcutaneous mouse xenograft models representing pancreatic cancer, triple-negative breast cancer, SCCHN and non-small cell lung cancer (NSCLC). All models showed significant levels of phosphorylated HER3, indicative of active HER3 signaling; therefore, growth of these tumors was suspected to depend at least in part on the HER3 pathway. These subcutaneous xenograft models contain no or very few immune effector cells at the tumor site; therefore, these models reflect mostly anti-tumor efficacy mediated via HER3 signaling inhibition with little or no contribution from ADCC.

RG7116 demonstrated dose-dependent TGI in a BxPC3 mouse xenograft model (Figure 4A). Intraperitoneal doses of 0.3–25 mg/kg RG7116 were highly efficacious, achieving >90% TGI compared with controls. Only partial TGI was achieved with 0.1 mg/kg RG7116, indicating suboptimal dosing. Phospho-HER3 levels were markedly reduced in tumor tissue obtained from mice treated with RG7116 at doses ≥0.3 mg/kg compared with controls (Figure 4B). Only the suboptimal dose (0.1 mg/kg RG7116) failed to inhibit HER3 phosphorylation completely. When explanted tissue was examined by
immunohistochemistry, the efficacious doses of RG7116 appeared to down-modulate membrane HER3 compared with controls and tumors treated with 0.1 mg/kg RG7116 (Figure 4C), thus confirming the Western blotting results.

Single-agent RG7116 induced potent TGI in HER3-positive human NSCLC models (Figure 5). Many of these models show resistance to established therapies, including anti-HER therapies (see Supplementary Material). Treatment with 4–6 cycles of weekly RG7116 at doses of 10–25 mg/kg resulted in strong TGI in 5/6 squamous NSCLC models, including tumor stasis or complete remission in 3/6 (Figure 5A). Figure 5B shows an example (LXFE722, squamous NSCLC) in which complete remission was achieved. In the LXFE646 model, where single-agent RG7116 did not inhibit tumor growth, tumor stasis was achieved when RG7116 was combined with cetuximab (anti-HER1, data not shown). Substantial TGI (>50%) was also observed in 5/10 adenocarcinoma NSCLC xenograft models (Figure 5A).

c-MET expression and KRAS mutation status are known for all the NSCLC tumor models. The anti-tumor efficacy of RG7116 mediated by HER3 signaling inhibition was low in the three adenocarcinoma cell lines that overexpress c-MET (HCC827, Lu7397 and NCI-H441), whereas TGI of >50% was seen in two KRAS-mutant models (A549 and LXFA983). No TGI was seen with NCI-H441, a KRAS-mutant lung cancer cell line that also overexpresses c-MET.

When combined with anti-HER1 (RG7160) or anti-HER2 (pertuzumab) antibodies, RG7116 efficacy was enhanced in models where HER1 (FaDu; Figure 5C) or HER2 (MAXF449; Figure 5D) is the preferred heterodimerisation partner, respectively. Combination treatment led to long lasting and complete tumor regression in both instances.

**In vitro and in vivo ADCC**

In addition to inhibiting tumor growth via blockage of HER3 and subsequent downstream signaling, RG7116 also demonstrated enhanced cell killing via ADCC. Using high-affinity (V158)-FcγRIIIa-expressing NK92 NK cells as effector cells and T-47D breast cancer cells
as target cells (E:T ratio 5:1), cell killing with RG7116 was markedly enhanced compared with its non-glycoengineered version (Figure 6A). At RG7116 concentrations >100 ng/mL, specific cell killing of 65% was achieved.

The influence of HER3 receptor density on the ADCC potential of RG7116 was investigated in vitro using T-47D cells (~21,800 receptors per cell [rpc]) and a series of A549 cell lines recombinantly expressing HER3 at densities of 1,800–34,200 rpc. These experiments clearly demonstrated a positive correlation between increased HER3 receptor density and susceptibility to RG7116-mediated ADCC (Figure 6B).

The A549 subcutaneous model has very poor immune infiltration and therapeutic efficacy will reflect only HER3 signaling inhibition but not Fc-related effector functions. In this model, RG7116 achieved identical TGI to that seen with the non-glycoengineered version (data not shown). To investigate the additional anti-tumoral impact of Fc glycoengineering of RG7116 in vivo, a more realistic A549 lung orthotopic xenograft model characterized by significant infiltration of macrophages (40) was used. SCID-beige mice (n=15 per group) i.v. injected with A549-B34 cells (14,400 rpc) were treated with weekly i.p. vehicle, glycoengineered RG7116 or non-glycoengineered RG7116 once scout animals had developed lung tumors. Median survival was significantly prolonged in animals treated with the glycoengineered form of RG7116 (92 days, 95% CI=78–99 days) compared with non-glycoengineered RG7116 (75 days, 95% CI=68–82; p=0.0001) or vehicle (57 days, 95% CI=54–61 days; p=0.0004; Figure 6C).
Discussion

Because of its lack of intrinsic kinase activity and its inability to homodimerize, HER3 requires other HER family members as heterodimerization partners for signaling. This may explain why there are no known tumors with HER3 gene amplification or HER3 “driver mutations”. Until recently, HER3 was not considered a target for immunotherapy of cancer (41), despite the fact that it is the only HER family member that can very effectively activate PI3 kinase signaling, a key pathway for cell proliferation and survival. However, this view changed when it became apparent that heterodimers of HER3 with HER1 and HER2 are the most active signaling dimers within the HER family (6,42) and also offer a major mechanism by which HER-driven tumors can escape from targeted therapy (15-18). HER3 is highly regulated and compensatory increases in transcription, translation, protein stability, duration of its tyrosine phosphorylation, cell surface localization, and heterodimerization have been implicated as key escape mechanisms underlying the resistance to other HER-targeting therapies (41); the latter at least in part through induction of autocrine ligand production.

In order to counter these multiple compensatory mechanisms, we generated an anti-HER3 antibody that prevents binding of HRG to the HER3-ECD (IC50: 0.13 nM) and binds with high affinity (t1/2-diss =131 minutes) to HER3-ECD. Crystallography data indicated that RG7116 binds HER3 in the inactive, tethered state, in which subdomains II and IV interact. However, this may also reflect the best uniform monomeric state suitable for crystallization under the experimental conditions investigated (pH 6). The inactivate receptor conformation seems to favor engagement of the HER3 kinase domain in homotypic interactions that further increase the barrier to ligand independent activation by sequestering HER3 into signaling-incapable receptor clusters and by covering in trans the C-terminal tail containing the tyrosine phosphorylation sites necessary for PI3 kinase recruitment (43).

Superimposition of the apo-HER3-ECD structure with the one bound by Fab(RG7116) showed no significant conformational changes. Thermodynamic analyses confirmed that the
binding of RG7116 to the HER3-ECD is a fully enthalpy-dominated interaction (20). The small activation enthalpy $\Delta H^{\ddagger}$ suggests that not much energy has to be spent on the disruption of non-covalent RG7116 intramolecular bonds in order to allow the new formation of intermolecular-RG7116/HER3-ECD forces. There is a negative “entropic burden” for the interaction, suggesting that no substantial solvent displacement, amino acid side chain movements or any major changes in the degrees of freedom of the interacting reactants take place. No induced fit mechanism could be identified. The supposed key-and-lock binding mechanism is typical for a fully mature antibody antigen interaction (44). In this way, RG7116 behaves similar to cetuximab, where the EGFR interaction is also enthalpy-dominated.

Since there is no crystal structure of HER3 in complex with one of its natural ligands reported so far, we cannot definitively say whether RG7116 occludes the ligand binding site on HER3 in a similar fashion as cetuximab does on HER1. While tether stabilization is an attractive model for the MoA of RG7116, other mechanisms should not be ruled out. The localization of the epitope recognized by RG7116 is such that is not clear why antibody binding would interfere with an extended conformation. Furthermore, the tethered species may even be capable of ligand-independent interaction through an alternative interface. Kani et al. also claim that the “tethered” conformation of HER3 cannot be completely discarded for ligand binding. HRG is assumed to bind to regions on domain 1 and domain 3 of HER3 in a fashion similar to the EGF-HER1 interaction (37;45). Therefore, we compared our HER3-ECD::Fab(RG7116) complex structure with a previously reported EGFR::EGF complex structure (46). Interestingly, the protein chains of EGF and Fab(RG7116) at least partly overlap in space, when both structures are aligned by the L domain-like $\beta$-barrel of ECD subdomain 1. Therefore, it is tempting to speculate that RG7116 might inactivate HER3 not only allosterically by locking the unliganded ECD conformation, but also by sterically blocking access to the ligand binding site. In agreement with this, we have seen competition between HRG and RG7116 for binding to the HER3 ECD in the HRG displacement assay (Figure3A) and in Biacore experiments (unpublished observations).
The high affinity of RG7116 for the unliganded HER3-ECD, and high complex stability of HER3-bound RG7116, translated into very potent HER3 signaling inhibition as demonstrated by almost complete abrogation of HER3 phosphorylation and AKT activation in several cancer cell lines \textit{in vitro}. Importantly, inhibition of HER3 activation by RG7116 was demonstrated both in the presence and absence of HRG ligand. Downregulation of membrane HER3 upon RG7116 treatment was consistently observed, further contributing to its inhibition of HER3 signaling \textit{in vitro} and \textit{in vivo}. HER3 downregulation by ligand-blocking antibodies has been reported before for α-HER3-ECD antibody (47) and in some cancer cell lines with MM-121 (48). The potent and complete blockage of HER3 activation by RG7116 resulted in substantial TGI in several mouse tumor xenograft models, including some complete remissions. This efficacy was achieved in models that were non-responsive to other HER therapies (cetuximab, erlotinib, gefitinib), other targeted therapies, and chemotherapeutic agents. The robust efficacy seen with RG7116 monotherapy in multiple preclinical models underscores the importance of HER3-containing heterodimers for tumor cell proliferation and survival, since RG7116 therapy does not block signaling by homodimers of the corresponding HER3 dimerization partner.

HER3 has recently emerged as a major cause of treatment failure for HER-targeted therapies; therefore, combining anti-HER3 therapies with HER1 and HER2 targeting agents is a clinically promising strategy. Combination treatment with cetuximab and the HER3 antibody MM-121 led to a more dramatic and sustained response than treatment with cetuximab alone (48). A very potent monospecific molecule like RG7116 will allow a free choice for tailoring combination regimens with the best partner agent for different patients and different tumor entities. We observed very long-lasting tumor regression of FaDu and LXFE646 xenografts by combining RG7116 with RG7160 or cetuximab and of MAXF449 xenografts by combining RG7116 with pertuzumab. Delayed resistance formation under a combination treatment with cetuximab and the HER3 antibody MM-121 was recently also shown by Schoeberl \textit{et al.}, 2010 (48). Despite high levels of HER3 expression and
activation, some tumors were not responsive to RG7116 monotherapy; tumors with high levels of total or activated c-MET were resistant to HER3 targeting. These tumors could be treated by a combination of RG7116 and an anti-c-MET antibody in the future. Even more challenging will be the therapy of tumors such as NCI-H441 that are characterized by high levels of HER3 expression, but functional uncoupling of the PI3K pathway from HER3 signaling (in this case due to constitutive c-MET activation) and constitutive MAPK pathway activation by RAS.

Antibody-mediated activation of NK cells and macrophages contributes significantly to tumor cell killing in patients (49). RG7116 is glycoengineered for enhanced binding of the Fc portion to FcγRIIIa on human immune effector cells such as NK cells. This additional mode of action can counteract the potential development of resistance to HER3 signaling inhibition and will broaden its therapeutic scope through inclusion of tumors that have sufficiently high HER3 surface levels but do not depend on HER3 signaling (e.g. due to c-MET upregulation). The influence of glycoengineering on the ADCC potential of RG7116 was clearly demonstrated in vitro (Figure 6). As murine NK cells lack FcγRIV (the murine homolog of human FcγRIIIa), the contribution of immune effector cells to RG7116’s MoA in vivo is limited to murine monocytes and macrophages (which do express FcγRIV). Using an orthotopic lung xenograft model of A549 which shows high levels of macrophage infiltration (40) we demonstrated a clear survival benefit of treatment with a glycoengineered versus non-glycoengineered RG7116 variant. HER3 is an ideal target for a glycoengineered antibody, since it is completely absent from peripheral blood cells and endothelium and therefore is unlikely to cause infusion reactions seen with glycoengineered antibodies against other targets. The recombinant cells used for the in vivo efficacy studies expressed relatively high levels of HER3 (~14,400 rpc), albeit less than the endogenous level seen in tumor cell lines like T-47D. Based on the in vitro ADCC assay results, a minimum expression level of ≥5,000 molecules/cell is probably required for a significant ADCC contribution to the therapeutic effect of RG7116.
In summary, RG7116 is an anti-HER3 antibody with a dual mode of action. On the one hand it stably binds to the inactive conformation of HER3 and downregulates it, resulting in complete HER3 signaling inhibition in vitro and superior efficacy in multiple preclinical tumor models. On the other hand its glycoengineered Fc portion allows efficient engagement of immune effector cells thereby contributing a signaling-independent component to its therapeutic efficacy in patients. RG7116 is currently in phase 1b clinical development.
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References


Figure Legends

Figure 1. Surface plasmon resonance data showing the thermodynamic profile of RG7116/HER3 (triangles) and cetuximab/HER1 (circles). RG7116 and cetuximab showed comparable thermodynamic profiles. The energy value is set to zero when reagents are separated from each other (A+B). During the association phase the kinetic energy overcomes the repulsive forces between the reactants and an activated complex is being formed (A-B, the transition state). From the transition state the molecules can dissociate back to A+B or can further transform into the final equilibrium complex (AB). (Left) The standard association free binding enthalpy ($\Delta G^{\ddagger}_{\text{ass}}$) was 43 kJ/mol for RG7116 and 40 kJ/mol for cetuximab. The standard equilibrium free binding enthalpy ($\Delta G^\circ$) was -53 kJ/mol for RG7116 and -50 kJ/mol for cetuximab. (Middle) Both antibodies show standard activation enthalpies ($\Delta H^{\ddagger}_{\text{ass}}$; 28 kJ/mol for RG7116 and 31 kJ/mol for cetuximab). The standard equilibrium binding enthalpy ($\Delta H^\circ$) for RG7116 was -70 kJ/mol vs -64 kJ/mol for cetuximab. (Right) The standard entropic term of the association phase ($T\Delta S^{\ddagger}_{\text{ass}}$) was 15 kJ/mol for RG7116 and 9 kJ/mol for cetuximab and the standard equilibrium binding entropy ($-T\Delta S^\circ$) was 17 kJ/mol for RG7116 and 14 kJ/mol for cetuximab. Within the error of the measurements, both antibodies behave in thermodynamically similar mode of actions.

Figure 2. Crystal structure of the HER3-ECD::Fab(RG7116) complex. (A) Structure of HER3-ECD (orange) in complex with Fab(RG7116) (heavy chain (HC) - blue, light chain (LC) – cyan) in ribbon representation with highlighted secondary structures. Fab(RG7116) binds to domain 1 of HER3-ECD. (B) Mapping of the Fab(RG7116) epitope on HER3-ECD domain 1 (surface in orange). Residues interacting with the Fab(RG7116) HC (light blue), LC (cyan) or both HC and LC (dark blue) are labeled. (C) Detailed view of the HER3-ECD::Fab(RG7116) interface region. Fab(RG7116) binds to domain 1 of HER3-ECD via hydrophobic but also polar and electrostatic interactions which involve residues from HC CDR1, CDR2, CDR3 and LC CDR1 and CDR3. Left panel: Interactions between HC CDRs (dark blue) and Her3-ECD domain 1 (orange). Right panel: Interactions between LC CDRs (cyan) and Her3-ECD domain 1 (orange). The Fab(RG7116) backbone region is colored in gray. Ordered water molecules are depicted as spheres (light red).

Figure 3. RG7116-mediated blockage of HER3 receptor activation in vitro. (A) RG7116 effectively displaced HRG with an IC50 of 0.13 nM when both compounds were co-incubated with immobilized HER3-ECD. Blockage of HRG-mediated HER3 activation by RG7116 on MCF7 and FaDu cells resulted in inhibition of HER3 phosphorylation (B) and AKT phosphorylation (C). Incubation of breast cancer cells lines with RG7116 in the absence of extraneous supplied heregulin resulted in downregulation of phospho-HER3 compared with untreated cell lines (D). FACS analysis revealed a decrease in the amount of membrane HER3 following incubation of T-47D breast cancer cells with 10 μg/mL RG7116, indicative of receptor internalization (E). RG7116 also inhibited proliferation of MDA-MB-175 cells grown in culture (F).
Figure 4. *In vivo* efficacy of RG7116 in SCID-beige mice (n=10 per group) bearing BxPC3 human pancreatic adenocarcinoma subcutaneous xenografts. (A) Mice were treated with five weekly i.p. doses of RG7116 beginning on Day 24 and tumor size measured by caliper. RG7116 at 0.3 mg/kg and above was highly efficacious and significantly inhibited tumor growth. Mice were sacrificed on day 56 and explanted tumor tissue was examined by Western blotting for expression of HER3 and phospho-HER3 (B) and for HER3 expression by immunohistochemistry (C). Efficacious doses of RG7116 inhibited HER3 phosphorylation and down-modulated membrane HER3 levels.

Figure 5. Tumor growth inhibition mediated by HER3 signal inhibition. (A) NSCLC cell lines or patient-derived tumor tissue fragments established as s.c. xenografts in SCID-beige or Balb/c nude mice (n=10 per group) and treated with 4–6 weekly doses of RG7116 (10–25 mg/kg). Substantial TGI was seen in squamous lung models (black bars), including complete remission in half the xenograft models examined, and in adenocarcinoma models (grey bars). * indicates c-MET high expressing models, † indicates KRAS-mutant models. (B) Time course for one of the patient-derived squamous tumor xenografts (LXFE772) in which complete remission was achieved with 6 cycles of 22 mg/kg RG7116. Tumors were undetectable by Day 95. Combination of RG7116 with other anti-HER antibodies enhanced efficacy. Complete tumor regression was achieved when RG7116 was combined with RG7160 (anti-HER1) in an s.c. SCCHN xenograft model (FaDu; Figure 5C) and with pertuzumab (anti-HER2) in an s.c. patient-derived tumor xenograft model MAXF 449 (Figure 5D).

Figure 6. ADCC activity of RG7116 *in vitro* and *in vivo*. (A) Glycoengineering of RG7116 resulted in superior *in vitro* ADCC efficacy compared with non-glycoengineered wildtype RG7116 using recombinant NK92 cells as effectors and T-47D cells as target cells (E:T ratio 5:1). (B) Efficacy of ADCC was associated with HER3 receptor density (E:T ratio 20:1). (C) In orthotopic located A549-B34 NSCLC xenografts in SCID-beige mice, treatment with 10–13 weekly doses of 25 mg/kg RG7116 significantly increased median survival (92 days) compared with mice treated with non-glycoengineered RG7116 (75 days; p=0.0001) or vehicle control (57 days; p=0.0004).
**Figure 3**

A. **Bound HRG (%)**

![Graph showing bound HRG (%) vs RG7116 concentration (nM)]

- **IC$_{50}$ = 0.13 nM**

B. **pHER3 inhibition in MCF7 cells (%)**

![Bar graph showing pHER3 inhibition in MCF7 cells (%) at different RG7116 concentrations (nM)]

- **MCF7 cells**
  - Positive control
  - 0.03 μg/mL (0.2 nM)
  - 0.1 μg/mL (0.7 nM)
  - 0.3 μg/mL (2 nM)
  - 1 μg/mL (7 nM)
  - 10 μg/mL (70 nM)

C. **AKT phosphorylation inhibition in MCF7 cells (%)**

![Graph showing AKT phosphorylation inhibition in MCF7 cells (%) vs RG7116 concentration (nM)]

- **IC$_{50}$ = 0.42 nM**

D. **Phospho-HER3 signal (%) in MDA-MB-175 cells**

![Bar graph showing phospho-HER3 signal (%) at different RG7116 concentrations (nM)]

- **MDA-MB-175 cells**
  - w/o RG7116
  - 0.1 μg/mL RG7116
  - 1 μg/mL RG7116

E. **Membrane HER3 (ratio vs unstained control)**

![Bar graph showing membrane HER3 (ratio vs unstained control) at different time points and RG7116 concentrations (nM)]

- **Control**
  - 0 min
  - 10 min
  - 30 min
  - 1 h
  - 5 h

F. **MDA-MB-175 cells tumor growth inhibition (%)**

![Graph showing tumor growth inhibition (%) vs RG7116 concentration (nM)]

- **EC$_{50}$ = 8.5 nM**
Figure 4

A. Tumor volume (mm$^3$), median ± IQR

B. HER3 and pHER3 expression levels across different treatments.

C. Representative images of tumor sections stained for HER3 and pHER3.
Figure 6

A

Specific cell lysis (%) vs. Antibody concentration (ng/mL)

- RG7116
- Non-glycoengineered RG7116

B

Specific cell lysis (%) vs. RG7116 concentration (ng/mL)

- A549-5H3 (34,200 rpc)
- T-47D (21,808 rpc)
- A549-B34 (14,429 rpc)
- A549-B35 (6,655 rpc)
- A549 (1790 rpc)

C

Surviving (%) vs. Study day

- RG7116
- Non-glycoengineered RG7116
- Vehicle
RG7116, a therapeutic antibody that binds the inactive HER3 receptor and is optimized for immune effector activation


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