A NOVEL GLYCOENGINEERED BISPECIFIC ANTIBODY FORMAT FOR TARGETED INHIBITION OF EGFR AND IGF-1R DEMONSTRATING UNIQUE MOLECULAR PROPERTIES

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Background: Bispecific antibodies are currently emerging as a promising new class of cancer therapeutics.

Results: The novel one-arm single chain Fab IgG bispecific antibody (XGFR) targeting IGF-1R and EGFR demonstrated potent signaling inhibition and enhanced ADCC induction.

Conclusion: XGFR has shown in vitro and in vivo anti-tumor activity in pancreatic, lung and colorectal mouse xenograft tumor models.

Significance: Rationale design can help to overcome low expression yields and impaired effector functions of bispecific antibodies.

ABSTRACT
In the present study, we have developed a novel one arm single chain Fab heterodimeric bispecific IgG (OAscFab-IgG) antibody format targeting the insulin-like growth factor receptor type I (IGF-1R) and the epidermal growth factor receptor (EGFR) with one binding site for each target antigen. The bispecific antibody XGFR is based on the knob-into-hole technology for heavy chain heterodimerization with one heavy chain consisting of a single chain Fab to prevent wrong pairing of light chains. XGFR was produced with high expression yields and showed simultaneous binding to IGF-1R and EGFR with high affinity. Due to monovalent binding of XGFR to IGF-1R, IGF-1R internalization was strongly reduced compared to the bivalent parental antibody leading to enhanced Fc-mediated cellular cytotoxicity. To further increase immune effector functions triggered by XGFR the Fc-portion of the bispecific antibody was glycoengineered, which resulted in strong ADCC activity. XGFR mediated inhibition of IGF-1R and EGFR phosphorylation as well as A549 tumor cell proliferation was highly effective, and comparable to a combined treatment with EGFR (GA201) and IGF-1R (R1507) antibodies. XGFR also demonstrated potent anti-tumor efficacy in multiple mouse xenograft tumor models with a complete growth inhibition of AsPC1 human pancreatic tumors and improved survival of SCID beige mice carrying A549 human lung tumors compared to treatment with antibodies targeting either IGF-1R or EGFR. In summary, we have applied rationale antibody engineering technology to develop a heterodimeric OAscFab-IgG bispecific antibody, which combines potent signaling inhibition with ADCC induction and results in superior molecular properties over two established tetravalent bispecific formats.

Introduction
Over the last years numerous bispecific antibody formats have been reported, and the approach to simultaneously modulate two molecular targets on a tumor cell or redirect immune effector cells to kill tumor cells may develop into an important therapeutic alternative to monoclonal antibodies. Notably, blinatumomab, a bispecific antibody targeting CD3 on T cells and CD19 on B cells in patients with acute lymphoblastic leukemia (1) has
demonstrated impressive clinical anti-tumor activity. Various other bispecific formats are currently in preclinical and clinical evaluation. One of the first published formats, the dual-variable-domain immunoglobulin (DVD-Ig) format is a tetravalent, dual targeting single agent generated by fusion of VH and VL domains to the N-terminus of a second antibody by a short amino acid linker (2). However, for this format, depending on the epitopes being targeted by the applied VH and VL domains, a loss of affinity for the inner variable domains was observed in one example caused by steric hindrance of ligand binding by the proximity of the outer variable domain (3). Another tetravalent bispecific format which has been extensively characterized is a monoclonal antibody carrying fusions of disulfide stabilized scFvs at the C-terminus of the heavy chain (4), (5). Recently, we have described a loss of ADCC activity with this bispecific format most likely due to steric hindrance caused by the attached scFv moieties preventing binding to the Fc gamma receptor IIIA on immune effector cells (6). These findings suggest that a thorough analysis of the consequences of antibody modification on the in vitro and in vivo properties of novel bispecific antibody formats is essential. Here, we rationally designed a heterodimeric one arm scFab-IgG antibody format targeting EGFR and IGF-1R, which combines potent signaling inhibition with effective ADCC induction through glycoengineering of the Fc region. Glycoengineered antibodies were produced using a method first described by Umana et al., 1999 (7). Glycosylation of human IgGs occurs in the Fc region at a conserved N-glycosylation site within the CH2 domain, where the asparagine 297 (Asn297) is linked to carbohydrates. The carbohydrate chain at this site contains a core of N-acetylglucosamine, mannose, galactose, sialic acid and fucose residues. Afucosylation or glycoengineering leads to an up to 100-fold increase in the affinity to FcγRIIIa receptors and subsequently to an increase in ADCC mediated cell death (8). Therefore, the antibodies in this study are absent of the core fucose at the Asn297 of the Fc region.

The receptor tyrosine kinases EGFR and IGF-1R contribute to tumor development and progression through their effects on tumor cell proliferation, inhibition of apoptosis and induction of angiogenesis (12), (13), (14). Several small molecules such as erlotinib or gefitinib as well as monoclonal antibodies such as cetuximab and panitumumab inhibit EGFR downstream signaling pathways and are approved for tumor treatment in the clinic (15), (16). We have reported the development of a glycoengineered and ADCC enhanced EGFR antibody termed imgatuzumab (GA201) which is currently in PhII clinical trials (17). In a phase I study imgatuzumab (GA201) was well tolerated and showed early signs of clinical efficacy in patients suffering from colorectal cancer (18). In addition, several monoclonal antibodies targeting IGF-1R such as AMG-479 or IMC-A12 are currently in clinical development either as monotherapy or in combination with chemotherapeutics or EGFR inhibitors (19), (20), (21), (22). We have developed an IGF-1R antibody termed R1507 that has been evaluated in combination with erlotinib in PhII clinical trials for advanced stage non-small-cell lung cancer (19). Both receptor tyrosine kinases contribute to tumor growth via activation of the PI3K-AKT and RAS-RAF-MAPK signaling pathways, and cross-talk between EGFR and IGF-1R signaling has been reported. Preclinical and clinical studies have shown that signaling through the IGF-1 receptor can overcome resistance to EGFR inhibitors (23), (24) and EGFR dependent signaling can confer resistance to IGF-1R inhibitors (25), (26), (27), (28), (29). Targeting EGFR and IGF-1R using bispecific antibodies to induce inhibition of tumor growth has been described in several publications, however, the applied bispecific antibody formats suffered from low production yields, inherent stability problems or lack of important anti-tumor effector mechanisms such as ADCC or inferior tumor cell proliferation inhibition compared to the parental antibodies. One example is a di-diabody with fusion of IFG-1R and EGFR targeting scFvs directly to the Fc domain, which showed a significantly reduced anti-proliferative activity compared to the respective monospecific EGFR antibody (26). In addition, a tetravalent bispecific antibody has been described devoid of ADCC effector functions containing a C-terminal
attachment of an IGF-1R scFv to the Fc part of an EGFR antibody (4). Another molecule lacking ADCC activity is a bispecific EGFR-IGF-1R inhibitor based on a human fibronectin scaffold, which has been PEGylated to increase serum half-life (30).

In the past, monospecific scFab antibodies have been mostly expressed in bacteria and yeasts and were found to be compatible for use in phage display (31). However, upon expression in HEK293 cells the formation of large amounts of oligomers was observed (32), (33), which renders this approach not viable for development of therapeutic proteins. In the present study, we rationally designed a bispecific heterodimeric and bivalent one arm scFab IgG (OA-scFab-IgG) antibody format based on the knob-into-holes technology (34) targeting EGFR and IGF-1R with distinct binding arms derived from the parental antibodies GA201 (EGFR) and R1507 (IGF-1R) (18;20). This novel antibody format combines robust expression and overcomes the light chain association issue in bispecific heterodimeric IgG antibodies (35). Furthermore, it combines potent signaling inhibition as well as reduced IGF-1R internalization with effective ADCC induction through glycoengineering of the Fc region. We also compared the OA-scFab-IgG XGFR with two other tetravalent bispecific antibodies in vitro and were able to improve impaired ligand binding and reduced Fc receptor activation of these formats. In several ADCC competent mouse xenograft models antibody XGFR demonstrated highly effective anti-tumoral activity.

**EXPERIMENTAL PROCEDURES**

**Generation of bispecific antibodies**

All antibody gene segments were generated by gene synthesis and cloned by unique restriction sites into pUC expression vectors. Bispecific and control antibodies were expressed by transient transfection of human embryonic kidney (HEK) cells growing in suspension. HEK cell culture supernatants were harvested 7 days after transfection and purified in two steps by affinity chromatography using Protein A-SepharoseTM (GE Healthcare) and Superdex 200 size exclusion chromatography. Glycoengineered antibodies were produced by co-transfection of the cells with two plasmids coding for the carbohydrate modifying enzymes β-1,4-N-acetyl-glucosaminyltransferase III and Golgi α-mannosidase II. XGFR was also expressed from stable Chinese hamster ovary (CHO) K1 cell lines engineered to constitutively overexpress the GA201 “knob” heavy and light chain and the R1507 OA-scFabAb “hole” heavy chain as well as recombinant β-1,4-N-acetyl-glucosaminyltransferase III and Golgi α-mannosidase II using the glutamine synthetase expression system (Lonza Biologics). The XGFR bispecific antibody was produced in a fed-batch fermentation process with the engineered CHO cells in a chemically defined animal component-free medium and was subsequently purified by protein A and ion-exchange chromatographic techniques. Fractions containing purified antibodies with less than 5% high molecular weight aggregates were pooled and stored in 6.0 mg/ml aliquots at -80°C.

**Biochemical and biophysical analysis of purified recombinant proteins**

The protein concentration of purified protein samples was determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence. Purity, antibody integrity and molecular weight of bispecific and control antibodies were analyzed by SDS-Page and CE-SDS using microfluidic Labchip technology (Caliper Life Science, USA). Aggregation of bispecific antibody samples was analyzed by high-performance size exclusion chromatography using a Superdex 200 analytical size-exclusion column (GE Healthcare) in 200 mM KH2PO4, 250 mM KCl, pH 7.0 running buffer at 25°C. The integrity of the amino acid backbone and the molecular weight of reduced bispecific antibody light and heavy chains was verified by Electrospray Q-TOF mass spectrometry after removal of N-glycans by enzymatic treatment with Peptide-N-Glycosidase F (Roche).

**Oligosaccharide analysis**

Oligosaccharides were enzymatically released from the antibodies by Peptide-N-Glycosidase F (Roche). A fraction of the PNGase F-treated sample was subsequently digested with
Endoglycosidase H (Roche). The released oligosaccharides were incubated in 150 mM acetic acid prior to purification through a cation exchange resin and analyzed using an Autoflex MALDI/TOF (Bruker Daltonics, Switzerland) in positive ion mode.

**Surface plasmon resonance (SPR) analysis**

All SPR experiments were performed using a Biacore T200 instrument with PBS/0.05% Tween20 (v/v). Standard amine coupling to ECD/NHS activated chip surfaces was performed as recommended by the provider (GE Healthcare). Antibodies were captured via anti-human IgG-Fc antibody. A CM5 chip was used for detection of EGFR binding with a ligand density of capture molecule of approx. 1000 RU and capture levels approx. 40-46 RU of the tested antibodies. A C1-Chip with low ligand density (approximately 200 RU, capture levels of Ab. approximately 6-10 RU) was used to achieve monovalent binding of the IGF1R dimer. Five increasing concentrations of the receptors were injected at a flow rate of 50 µL/min for 180 seconds association time and a dissociation time of 1200 s for EGFR and 1600 s for IGF-1R at 37°C. Final regeneration was performed after each cycle using 10 mM Glycine pH 1.75, contact time 60 secs and a flow rate of 50 µL/min. Kinetic constants were evaluated by fitting the association and dissociation phase of the analyzed interaction with a Langmuir 1:1 binding model (RI = 0) using usual double referencing (FC1 reference surface with capture molecule and c = 0 nM) by Biacore evaluation Software.

**Cell culture**

A549 (ATCC), AsPC1 (ATCC), LS174T (ATCC) and NCI-H322M cells (National Cancer Institute) were cultivated in RPMI 1640 medium (PAA, Austria) supplemented with 10% fetal bovine serum (PAA) and 2 mM L-glutamine (Sigma, Germany) at 37°C and 5% CO₂.

**IGF-1R / EGFR phosphorylation assay**

A549 NCSLC cells (3 x 10⁵) were seeded in 96-well plates and starved in serum-free medium (1 mg/ml RSA, 10 mM HEPES, 1% Pen/Strep) for 2 hours. To assess phosphorylation, cells were first incubated with either bispecific or control antibodies for 30 min, followed by addition of 5nM IGF-1 (PreProtech) or 10nM EGF (PreProtech) for 10 min and final lysis in 100 µl/well lysis buffer (Cell Lysis Kit, BioRad). Samples were analyzed for EGFR or IGF-1R phosphorylation using the P-EGFR (Tyr) bead kit (Millipore) or P-IGF-1R (Tyr1131) bead kit (BioRad) combined with a phosphoprotein detection reagent (BioRad) by the Luminex detection system.

**Signaling pathway analysis**

LS174T cells (1.5 x 10⁶) were seeded in 6-well plates overnight and incubated with 1000 nM bispecific or control antibodies for 0.5, 6, 24 and 48 hours. Cells were lysed by addition of 10 ml lysis buffer (Cell Lysis Kit, BioRad). Samples were analyzed for PI3K (Bio-Plex Assay, Biorad) and MAPK pathway (WideScreen EpiTag Assay, Novagen) activation markers using the Luminex detection system.

**3D cell viability assay**

Tumor cells were seeded into 96-well poly-HEMA coated plates with increasing concentrations of bispecific or control antibodies and incubated for 7 days. Cell viability was determined using a CellTiterGlo® (Promega) assay according to manufacturer’s instructions.

**IGF-1R levels in cell lysates**

Tumor cells were cultivated in the presence of 50 nM concentrations of bispecific or control antibodies in standard culture medium for 24 hours at 37°C and 5% CO₂. Cells were then lysed with ice-cold cell signaling lysis buffer (Millipore) supplemented with protease inhibitors (Roche). Plates were stored at -20°C until further analysis. IGF-1R levels were detected using an IGF-1R sandwich ELISA using a biotinylated <IGF-1R>hu-1a-IgG (Roche) capture antibody and an <IGF-1Rβ> rabbit (Santa Cruz Biotechnology) detection antibody.

**Quantification of antibodies on the surface of tumor cells**

A549 cells were seeded into poly Hema-coated 6-well tissue culture plates to prevent adhesion of cells to the plastic surface for 3D-cultures. Coating was performed using 4% poly-HEMA (Polysciences) in EtOH and subsequently drying for at least 5 days at 37°C. After 4 hours of cell
seeding, Matrigel (BD) was added and spheroids were collected 24h later, washed and trypsinized to generate single cell suspensions. Subsequently, cells were washed twice with ice-cold medium and stained with 10µg/ml of PE-conjugated EGFR or IGF-1R antibodies (BD) or matching isotype controls (BD). Acquisition of data was performed using FACS Canto II and analyzed by FlowJo software. The number of antibodies bound per cell was determined using a Phycoerythrin Fluorescence Quantification Kit (BD) and receptor density per cell was calculated.

**In vitro ADCC assay**
Performed as previously described (6).

**In vivo studies**
Human pancreatic cancer AsPC-1-LUC cells were orthotopically inoculated into the pancreas of female SCID beige mice (1×10^6 cells / mouse). Groups of n=5 animals were treated with intraperitoneal injection of control antibody (Xolair, Roche) or the bispecific antibody XGFR on day 7, 14 and 21 after tumor inoculation at a 20 mg/kg dose. Orthotopic tumor growth of luciferase positive AsPC-1 xenografts was assessed by Bioluminescence Imaging using the IVIS Xenogen System on study day 7, 14, 21 and 27 after tumor inoculation. In the A549 lung tumor model, A549 cells were injected intravenously in the tail vein of female SCID beige mice. Groups of n=10-15 mice were treated once weekly by intraperitoneal injections of compounds or vehicle control starting on day 7 after tumor cell inoculation, when evidence of tumor growth was visible in the lungs of sacrificed scout animals. GA201 and R7072 (glycoengineered R1507) were applied at 10 mg/kg whereas XGFR was applied at 20 mg/kg to ensure equimolar concentrations of dosing between XGFR and GA201/R7072 combination therapy. In the LS174T colon carcinoma metastasis model, 3×10^6 human colorectal adenocarcinoma LS174T cells were inoculated into the splenic tissue of female SCID beige mice. Weekly intraperitoneal administration of vehicle or test compounds 20 mg/kg XGFR, 20 mg/kg XGFR-wt and 10 mg/kg AMG-479 (resynthesized according to patent information)/10 mg/kg cetuximab (Merck, Germany) was started on day 7 after tumor cell inoculation (n=10/group). Animals with severe clinical symptoms (e.g. body weight loss > 20%) were excluded according to animal welfare guidelines and classified as non-survivors. Survival data were analyzed using Kaplan-Meier curves and statistical analysis between treatment groups were assessed by Pairwise Log-Rank test.

**RESULTS**

**Design of a heterodimeric one arm scFab bispecific antibody targeting EGFR and IGF-1R (XGFR)**
The bispecific antibodies generated in this study are based on a human IgG1 isotype with heavy chains comprised of a variable VH domain and 3 constant domains CH1, CH2 and CH3. The corresponding light chains are composed of a variable VL domain and a constant C kappa domain. XGFR bispecific antibodies were assembled with an EGFR binding arm comprised of the GA201 light and heavy chain. The IGF-1R binding arm is composed of a single chain Fab-fragment (scFab) of R1507 with the light chain attached to the N-terminus of the VH domain by a thirty two amino acid glycine serine linker to form the second heavy chain (Fig 1A). Heterodimerization of the two heavy chains in this novel bispecific antibody format was achieved by application of the knobs-into-hole technology (34). The knob mutation (T366W) was introduced into the CH3 domain of the GA201 heavy chain, and three mutations to form a hole (T366S, L368A, and Y407V) were introduced into the CH3 domain of the scFab heavy chain of R1507. In addition, two cysteine residues were introduced (S354C on the knob and Y349C on the hole side) to form a stabilizing disulfide bond between the heterodimeric heavy chains. To compare the in vitro properties of the bivalent bispecific antibody XGFR with other bispecific formats, we generated two established tetravalent bispecific formats XGFR-DVD (dual variable domain-IgG) and XGFR2 (single chain Fv-IgG) (2;6). In the XGFR-DVD (Fig 1A), additional GA201 VH and VL variable domains were fused by a short peptide linker on the N-terminus of heavy and light chain of the R1507 antibody. XGFR2 (Fig. 1A) was constructed with disulfide stabilized GA201 scFvs fused to the C-terminus of the R1507 heavy chains by a 10 amino acid (G[S]_2) linker (6). The VH and
VL region of the GA201 scFvs were tethered by a glycine serine peptide (G
S
4
S
4
) linker and stabilized to eliminate aggregation by the previously described VH44 and VL100 disulfide bond (36), (37).

Expression and purification of XGFR bispecific antibodies
Monoclonal antibodies GA201 and R1507 as well as XGFR bispecific antibodies were produced by transient expression in HEK293 cells. The parental antibodies as well as XGFR, XGFR-DVD and XGFR2 were purified to homogeneity by protein A and size exclusion chromatography from cell culture supernatants as demonstrated by SDS-PAGE analysis under reducing conditions (Fig. 1B). Reduced SDS-PAGE analysis of XGFR shows a 74.7 kDa R1507 scFab hole heavy chain band, a 49.3 kDa GA201 knob heavy chain and a 23.4 kDa light chain (Fig. 1B). Upon transient expression, purification yields of the novel one arm scFab bispecific antibody XGFR (20.6 mg/L) were similar to the GA201 and R1507 parental antibodies with yields of 24.0 and 23.6 mg/L, respectively (Table 1). XGFR2 with C-terminal fusion of scFvs showed approximately 2-fold reduced expression, whereas attachment of N-terminal VDs led to an 18-fold reduction in expression of XGFR-DVD compared to the parental antibodies (Table 1).

Generation of stable CHO cell lines
Most therapeutic antibodies in clinical development are currently produced in stable CHO cell lines. Therefore, the manufacturing scalability of the novel bispecific antibody format XGFR was evaluated in CHO cells. GA201 knob heavy and light chain and the R1507 OAscFAb hole heavy chain as well as recombinant β-1,4-N-acetyl-glucosaminyltransferase III and Golgi α-mannosidase II were over expressed in CHO K1 cells using a glutamine synthetase expression system. A total number of 415 clones were analyzed for IgG titer and afucosylation, and 26 clones were selected for generic fed batch production. Several stable production CHO clones with yields of 2-3 g/l and afucosylation levels above 70% were identified and demonstrate the excellent manufacturing feasibility of the XGFR bispecific antibody.

Characterization of the by-product profile of XGFR
The product quality of purified XGFR was further analyzed by analytical size exclusion chromatography, CE-SDS and mass spectroscopy (MS) analysis. Initially, analytical size exclusion chromatography was used to evaluate the levels of high molecular weight aggregates and XGFR was found to be 99 % pure after protein A and SEC purification (Fig. 2A). Product homogeneity of the GA201 light and heavy chain and the OAscFAb R1507 heavy chain was confirmed by CE-SDS under reducing conditions and a single monomer peak of XGFR was found under non-reducing conditions (Fig. 2B). The expected molecular weight of 147.4 kDa of XGFR was confirmed by mass spectroscopy. In addition, the potential formation of knob-into-hole antibody byproducts such as hole-hole and knob-knob heavy chain homodimers, half antibodies lacking a knob or hole heavy chain and ¾ antibodies lacking the GA201 light chain was analyzed and none of the mentioned byproducts was detectable by MS analysis (Fig. 2C).

SPR analysis of the XGFR bispecific molecule binding affinities
The bispecific XGFR antibodies were analyzed for association rate constants (ka), dissociation rate constants (kd), and equilibrium constants (K
D
) using surface plasmon resonance analysis. Firstly, the EGFR extracellular domain was coupled to the chip surface and XGFR antibodies were injected, followed by the IGF-1R extracellular domain, which confirmed that all of the bispecific antibodies could simultaneously bind to both receptors (data not shown). All XGFR bispecific molecules could bind to EGFR within the same low nanomolar range as the parental antibody GA201 (Table 2). Binding to the IGF-1R extracellular domain was strongly dependent on the design of the bispecific antibodies. XGFR and XGFR2 showed K
D
 values of 4 and 6 nM, which are similar to the parental R1507 K
D
 of 5 nM. In contrast, XGFR-DVD with a rigid 5 amino acid attachment of GA201 variable domains to R1507 completely lost the ability to bind IGF-1R and therefore was not further evaluated in vitro and in vivo (Table 3).
XGFR bispecific antibodies maintain high levels of IGF-1R cell surface expression

Previous studies have demonstrated that IGF-1R and EGFR targeting antibodies can reduce receptor cell surface expression by inducing internalization and subsequent degradation in the endosomal-lysosomal cell compartment. While GA201 only induces minimal EGFR degradation in vitro (data not shown), the IGF-1R antibody R1507 induces strong receptor degradation of 87% in A549 non-small cell lung cancer cell lysates after 24 hours (Figure 3A). As bispecific XGFR antibodies should simultaneously bind both receptors on the cell surface and may cross-link the two receptor tyrosine kinases, we next investigated whether binding by a bivalent or tetravalent BsAb format affects total IGF-1R and EGFR levels in cell lysates. EGFR degradation following XGFR incubation was similar compared to the parental antibody GA201 (data not shown). IGF-1R levels in lysates of cells treated with XGFR were 92.7% of untreated controls, whereas treatment with the parental IGF-1R antibody R1507 or the combination of R1507 + GA201 induced receptor degradation to levels of 13.0% after 24 hours (Figure 3A). The tetravalent bispecific antibody XGFR2 induced IGF-1R internalization and degradation similar to R1507 (Figure 3A). In agreement with these findings, high levels of XGFR on the cell surface were still detectable after 24 hours incubation by FACS analysis (Fig 3B). Incubation of H322M cells with the parental IGF-1R antibody R1507 or the combination of R1507 + GA201 induced receptor degradation to levels of 13.0% after 24 hours (Figure 3A). The tetravalent bispecific antibody XGFR2 induced IGF-1R internalization and degradation similar to R1507 (Figure 3A). In agreement with these findings, high levels of XGFR on the cell surface were still detectable after 24 hours incubation by FACS analysis (Fig 3B). Incubation of H322M cells with the parental IGF-1R antibody R1507 or the combination of R1507 + GA201 induced receptor degradation to levels of 13.0% after 24 hours (Figure 3A). The tetravalent bispecific antibody XGFR2 induced IGF-1R internalization and degradation similar to R1507 (Figure 3A). In agreement with these findings, high levels of XGFR on the cell surface were still detectable after 24 hours incubation by FACS analysis (Fig 3B). In summary, a 13-fold increased antibody density of XGFR compared to R1507 and a 2.4-fold increased density towards the R1507/GA201 combination was observed on the cell surface of tumor cells after 24 hours incubation. As reduced receptor down-regulation and remaining antibody density on the cell surface may affect induction of effector cell mediated functions we next compared ADCC induction of the different BsAb formats.

Antibody-dependent cellular cytotoxicity of the XGFR bispecific antibodies

ADCC activity relative to the afucosylated parental antibodies R7072 (glycoengineered version of R1507) and GA201 combination was measured with H322M target cells and the NK92 effector cell line. XGFR showed a maximal killing efficiency of 75% with a highly potent IC50 of 7 pM and was slightly superior to the combination of the parental antibodies (Fig. 3C). The XGFR2 bispecific molecule exhibited significantly reduced ADCC activity. Here, the scFvs were attached to the C-terminal to the heavy chain, which results in a loss of ADCC activity (Fig. 3C). Interestingly, ADCC activity correlated with receptor density on the cell surface - GA201, which induces modest receptor internalization, showed more potent ADCC activity than R7072 (Fig. 3C) with strong receptor downmodulation (Fig. 3A) despite a similar receptor density of IGF-1R and EGFR on H322M cells (Table 4). Increased density of XGFR on the cell surface (Fig. 3B) translated into slightly enhanced ADCC induction compared to the parental antibody combination GA201 and R7202. In conclusion, the XGFR OAscFab bispecific bivalent antibody format was optimized for ADCC induction and clearly superior to the tetravalent XGFR2 scFv antibody.

Inhibition of EGFR and IGF-1R phosphorylation by XGFR bispecific antibodies in vitro

In order to evaluate the capacity of XGFR to inhibit IGF-1R and EGFR phosphorylation in comparison to the bivalent parental antibodies GA201 and R1507, we investigated the inhibition of EGFR and IGF-1R phosphorylation on A549 human NSCLC cells. The A549 cell line expresses high levels of both receptors on the cell surface (see Table 4). XGFR potently inhibited IGF-R phosphorylation with a maximum inhibition level of approximately 80% and an IC50 value of 0.35 nM (Fig. 4A). The combination of the parental antibodies with an active R1507 component resulted in a comparable IC50 of 0.42 nM indicating that IGF-1R phosphorylation inhibition by XGFR was similarly potent as the bivalent R1507 antibody in spite of being monovalent for the respective receptor. The bispecific tetravalent antibody XGFR2 also showed IGF-1R phosphorylation inhibition in a similar range compared to the parental antibody R1507 as we have published previously (6). Due to lack of binding to IGF-1R the XGFR-DVD bispecific antibody format was not tested for phosphorylation inhibition. EGFR phosphorylation was inhibited even more strongly by XGFR with a
 maximal inhibition of 100% and an IC50 value of 0.23 nM (Fig. 4B). Here, the combination of the bivalent antibodies with an active GA201 antibody resulted in IC50 of 0.18 nM and was comparable to XGFR (Fig. 4B). As expected, XGFR2 induced phosphorylation inhibition in a similar manner as the parental GA201 in the combination with R1507 (6). In summary, XGFR efficiently inhibits IGF-1R and EGFR dependent signaling in vitro and retains the potent signaling inhibition properties of the parental antibodies.

**Inhibition of downstream signaling pathways by XGFR bispecific antibodies in vitro**

In order to evaluate the capacity of XGFR to inhibit IGF-1R and EGFR dependent signaling pathways, LS174T colorectal carcinoma cells were incubated with XGFR, R1507 or GA201 and levels of phosphorylated IGF-1R, phosphorylated RAF1, phosphorylated AKT and phosphorylated ERK1 were analyzed. While XGFR inhibited IGF-1R phosphorylation comparable to R1507 (Figure 5A), inhibition of downstream signaling pathway components RAF1, AKT and ERK1 by XGFR was superior to the individual parental antibodies GA201 or R1507 (Figure 5B-D).

**Inhibition of 3D tumor cell proliferation mediated by XGFR**

Inhibition of IGF-1R and EGFR dependent signaling by XGFR also translated into potent inhibition of tumor cell viability in a 3D cell proliferation assay with NSCLC A459 tumor cells (Fig. 4C). XGFR inhibited A549 tumor cell growth by approximately 80% with an IC50 value of 0.11 nM after 7 days of incubation. Tumor cell proliferation inhibition mediated by XGFR was comparable to the combination of the parental antibodies R1507 and GA201, which inhibited A549 spheroid proliferation with an IC50 value of 0.08 nM (Fig. 4C). The tumor cell proliferation inhibition of XGFR2 was evaluated with NSCLC H322M cells and resulted in potent tumor growth inhibition within a similar range as the parental antibody R1507 and GA201 combination. XGFR-DVD was not tested for tumor cell proliferation inhibition as we have shown previously that single agent GA201 tumor cell proliferation inhibition was less efficient than the combined inhibition of EGFR and IGF-1R signaling (6).

**XGFR inhibits tumor growth in preclinical in vivo models**

To confirm the efficacy of XGFR in vivo, its anti-tumor activity was evaluated in several tumor models in SCID beige mice. Firstly, human pancreatic cancer AsPC-1 cells, which express high levels of EGFR and 6.4 fold lower levels of IGF-1R and on the cell surface (Table 4), were transfected with the luciferase gene and orthotopically inoculated into the pancreas of female SCID beige mice. Mice were treated with intraperitoneal XGFR or control antibody (omalizumab) injections on day 7, 14 and 21 after tumor inoculation at a dose of 20 mg/kg. Orthotopic tumor growth of luciferase positive AsPC-1 xenografts was assessed by bioluminescence imaging on study day 7, 14, 21 and 27. While signal intensity strongly increased over time in control animals, luminescence signal in XGFR treated animals remained low over the entire observation period, demonstrating potent anti-tumor activity of XGFR (Fig. 6A & B). Moreover, human A549 NSCLC cells were injected in the tail vein of SCID beige mice resulting in formation of orthotopic lung metastases. Treatment with weekly injections of the bispecific antibody XGFR at 20 mg/kg resulted in statistically significant increased survival compared to treatment with the single agent GA201 or glycoengineered R1507 (R7072) (p < 0.0001). XGFR treatment was as efficacious as the combination of GA201 and R7072, confirming the strong anti-tumor activity of XGFR (Fig. 7A). XGFR was also evaluated in the LS174T colon carcinoma metastasis model, where LS174T cells were inoculated in the spleen of SCID beige mice as described previously (17). Here, the in vivo effects of afucosylation of the Fc region were assessed by comparing XGFR to XGFR-wt (non-glycoengineered version of XGFR). In addition, a combination of the therapeutic EGFR antibody cetuximab with the IGF-1R antibody AMG-479 (resynthesized based on patent information) was evaluated. Treatment with each bispecific antibody at a weekly dose of 20 mg/kg resulted in significant improvement of survival compared to vehicle control (p = 0.000005 or 0.00001, respectively). Survival of animals treated with XGFR was significantly improved compared to XGFR-wt (p=0.033) as well as the cetuximab/AMG-479 combination (p = 0.0005) in
DISCUSSION

We have constructed a novel afucosylated heterodimeric one arm scFab bispecific IgG1 antibody with enhanced ADCC activity that is capable to simultaneously bind IGF-1R and EGFR, and induces potent inhibition of receptor signaling as well as tumor cell proliferation. In the present study, we have performed a detailed analysis of protein expression, receptor binding and down modulation, phosphorylation inhibition, ADCC activity, as well as, in vivo efficacy in ADCC competent mouse tumor models.

The bispecific antibody XGFR uses a single chain Fab with a glycine serine linker on one arm to overcome the light chain mispairing issue in bispecific heterodimeric IgG antibodies and achieves heterodimerization of two antibody heavy chains with different binding specificities applying the “knob-into-hole” technology. The parental antibody GA201 (EGFR) was introduced into the bispecific antibody as knob heavy and light chain and the R1507 (IGF-1R) antibody as OAscFab hole heavy chain. The introduction of a scFab arm allowed us to rapidly generate a bispecific antibody without the need for variable region engineering such as disulfide stabilization usually applied in tetravalent scFv antibody formats or time and labor intensive optimization by phage display in the “two in one” antibody technology (38), (39). The only modifications to the native structure of an IgG antibody were the introduction of the “knobs into hole” mutations into the CH3 domain of each heavy chain (34) and the addition of a 32 amino acid glycine serine linker between the N-terminus of the VH region and the C-terminus of the Ck domain in the IGF-1R binding arm. The “knob-into-hole” technology is currently evaluated in phase II clinical trials in an one-armed 5D5 anti-cMet antibody (40) as well as in an Ang-2-VEGF CrossMAb in phase I clinical trials and no negative effect on the safety profile of the engineered antibodies was reported (41). Moreover, the glycine serine recognition motif has not been identified for any of the known MHC alleles (42) and immunogenicity of a 15 amino acid glycine serine linker has been examined in the clinic for a bispecific T cell engaging molecule with no adverse events being described (43), indicating that the glycine serine linker is currently the best available peptide linker to tether scFv or scFab molecules in the engineering of antibodies. The described OAscFab XGFR bispecific antibody showed no aggregation when analyzed by analytical SEC in agreement to Lee and colleagues, who published a monospecific scFab antibody targeting the tumor associated glycoprotein (TAG)-72 and expressed this molecule in mammalian CHO cells in 1999 (42). Interestingly, when Dübel and colleagues characterized scFab fragments expressed in E. coli high amounts of multimers and dimers were found as final product (31) leading to the expression of monospecific single chain IgG molecules in HEK293T cells with the formation of large amounts of oligomers (32). Here, we have combined the scFab technology with “knob-into-hole” heterodimerization of two distinct heavy chains to generate a novel bispecific antibody with excellent production yields of 2-3 g/L in CHO cells and low formation of multimers.

For comparison we also generated XGFR2 a full length IgG1 antibody comprised of an IGF-1R master molecule with EGFR disulfide stabilized scFv moieties attached to the CH3 domain by a (G4S)₄ linker (6) and a DVD-Ig molecule based on a R1507 IGF-1R master antibody with GA201 VH and VL domains attached to the N terminus by a five amino acid linker as described previously (2). In this study, the OAscFab XGFR and the scFv containing derivative XGFR2 showed transient expression levels which correlated with the parental antibodies, however, the DVD-Ig molecule showed significantly reduced expression levels in the HEK293 transient expression system. In addition, the XGFR-DVD molecule with its short five amino acid linker as described previously (2). The OAscFab format clearly showed the advantage of converting an existing antibody in a bispecific molecule without loss of target antigen binding.
and evaluation of a linker optimization strategy. Notably, monovalent binding of target antigens EGFR and IGF-1R by XGFR resulted in highly potent inhibition of receptor phosphorylation and tumor cell proliferation comparable to the bivalent parental antibodies GA201 and R1507. Other IGF/EGFR bispecific antibodies such as the EGFR/IGF-1R di-diabody showed a 25-fold reduced tumor cell proliferation inhibition in comparison with their parental antibody combination despite tetravalent binding of the target antigens, presumably due to a reduced binding affinity for EGFR (26). The most striking property of the OAscFab bispecific format was the significantly reduced IGF-1R internalization and subsequent degradation in tumor cells compared to the parental antibody R1507 or the tetravalent bispecific antibody XGFR2. Here, we have shown by FACS analysis that reduced IGF-1R down-modulation on tumor cells leads to an increased antibody density of XGFR on the tumor cell surface. Interestingly, the increased amounts of XGFR on the tumor cell surface translated into slightly enhanced ADCC induction compared to the EGFR targeting antibody GA201 with low receptor internalization and strongly increased activity over the IGF-1 R targeting antibody R7202 with high receptor down modulation. In conclusion, we report for the first time a glycoengineered bispecific antibody format optimized for ADCC induction with unique effects on IGF-1R down modulation. In addition, we demonstrate clear superiority of the OAscFab format compared to the tetravalent XGFR2 scFv antibody, which shows a loss of ADCC activity in vitro most likely due to steric hindrance of FcγIII receptor binding on effector cells by C-terminal attachment of scFvs on the C-terminus of the CH3 domain of the Fc region (6). Despite the lack of internalization the OAscFab XGFR showed potent IGF-1R and EGFR phosphorylation inhibition in vitro attributed to the inhibition of receptor dimerization and subsequent activation of the kinase domain. Inhibition of AKT, ERK1 and RAF1 as representative signaling components of IGF-1R/EGFR downstream signaling pathways was superior with XGFR compared to individual EGFR (GA201) or IGF-1R antibodies. Furthermore, XGFR retained the ability to inhibit the growth of tumor cells comparable to the parental antibodies indicating that receptor internalization, at least in vitro, was not required or essential for tumor growth inhibition. Potent in vivo efficacy of the bispecific OAscFab antibody XGFR was shown in several mouse xenograft models. XGFR treatment led to complete tumor growth arrest in the orthotopic human pancreatic carcinoma model AsPC-1-luc and an improvement of survival in the orthotopic lung carcinoma model A549 and the colon carcinoma metastasis model LS174T. In vivo efficacy of OAscFab XGFR was shown to be comparable to the parental antibody combination confirming the full retention of anti-tumor activity of GA201 and R7072 in the A549 mouse xenograft model. The mouse model reflects the in vitro experimental data of XGFR showing similar phosphorylation and tumor cell proliferation inhibition and slightly improved ADCC activity compared to the parental antibody combination. However, the combination of targeting both receptors in a single molecule is clearly more potent than dosing of an individual monospecific antibody in vivo. The contribution of glycoengineering to anti-tumor efficacy of XGFR in vivo was investigated in the LS174T colon carcinoma model in SCID beige mice. Anti-tumor activity of XGFR was improved over the non-glycoengineered antibody XGFR-wt or a combination of the therapeutic EGFR antibody cetuximab with the IGF-1R antibody AMG-479, showing the therapeutic potential of combining IGF-1R/EGFR signalling inhibition with potent ADCC induction in a bispecific antibody format. This unique feature provides XGFR an important advantage over other bispecific antibodies targeting EGFR and IGF-1R such as a tetravalent bispecific antibody with IGF-1R scFvs fused to the Fc part of an EGFR antibody devoid of ADCC effector functions (4) or a fibronectin based bispecific scaffold (30), which lack direct recruitment of immune cells through the Fc γ receptor. Furthermore, bispecific antibody design with enhanced ADCC induction may lead to an important improvement of cytotoxic activity for targeting tumors with mutated signaling pathways such as K-Ras, where ADCC can aid to the killing of resistant tumor cells. Recently, we have shown that the glycoengineered EGFR antibody GA201 significantly enhanced in vitro induction of ADCC compared with cetuximab and in vivo efficacy in a series of mouse xenograft models (17). Generation
of the bispecific antibody XGFR could even further increase the potent activity of GA201 against tumors, which express EGFR and IGF-1R on the cell surface.

In summary, we have demonstrated that rationale antibody design can help to overcome technical hurdles related to bispecific antibody design and generated a bispecific anti-IGF-1R/EGFR antibody XGFR, which induces more potent phosphorylation inhibition of downstream signalling mediators RAF-1, AKT and ERK1 than the parental antibodies. In addition, IGF-1R internalization was reduced after XGFR treatment leading to higher receptor levels at the cell surface and enhanced ADCC activity compared to the combination of GA201 and R1507. Antitumor activity of afucosylated XGFR was superior to individual anti-EGFR and anti-IGF-1R antibodies, and combined therapeutic EGFR/IGF-1R antibody Cetuximab/AMG-479 (resynthesized) treatment in various mouse tumor models. Therefore, XGFR may be a promising new therapeutic option for the treatment of pancreatic, pulmonary or colorectal cancer.

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REFERENCES


bispecific inhibitors of epidermal growth factor receptor and insulin-like growth factor-I receptor. *MAbs.* 3 (1), 38-48


FIGURE LEGENDS

Figure 1. Design of XGFR (EGFR/IGF-1R) bispecific antibodies. A, Schematic diagram of the one arm single chain Fab bispecific antibody XGFR, the XGFR2 antibody with C-terminal attachment of disulfide stabilized scFvs and the dual V domain (DVD) antibody XGFR-DVD. All VH and VL domains in the bispecific antibodies are derived from the parental antibodies GA201 (EGFR, red and yellow) and R1507 (IGF-1R, blue and light blue). The R1507 light chain in the XGFR molecule was fused by a 32 amino acid (G4S)6GG linker (green) to the N-terminus of the VH domain. Dimerization of the two different heavy chains in the XGFR antibody was facilitated by the knob-into-hole mutations (orange) in the CH3 domain and an additional disulfide bond (black bar). The XGFR molecule design is based on the R1507 master antibody with a GA201 scFv fused by a (G4S)2 connector (green) to the C-terminus of the CH3 domain. VH and VL domains are joined by a (G4S)4 linker (green). In the XGFR-DVD molecule the GA201 VH and VL domains were fused to the R1507 antibody by a ASTKGP (pink) heavy chain and a TVAAP (purple) light chain linker. (B) SDS-PAGE of bispecific XGFR antibody variants under reducing conditions after purification by Protein-A and size exclusion chromatography (SEC).

Figure 2. Biochemical and biophysical analysis of purified XGFR. A, Analytical size exclusion chromatography (SEC) was used to estimate the presence of aggregates in the one arm scFab XGFR molecule after protein A and SEC purification. The chromatogram represents a 20 µg injection. (B) Purity, antibody integrity and molecular weight of XGFR was further characterized by CE-SDS under reducing (red) and non-reducing (blue) conditions. (C) MS analysis confirmed sequence integrity of XGFR and no by products such as hole-hole dimers, knob-knob dimers, half antibodies or ¾ antibodies lacking the GA201 light chain were detectable.

Figure 3. Reduction in IGF-1R protein levels after antibody treatment on non-small cell lung cancer cells and induction of ADCC. A, Reduction in total IGF-1R protein levels was analyzed by IGF-1R ELISA 24 hours after incubation of A549 cells with 50 nM XGFR, XGFR2, GA201, R1507 and the R1507/GA201 combination and subsequent cell lysis. The mean of triplicate experiments are shown. (B) FACS analysis of XGFR, R1507 and R1507/GA201 on the surface of A549 cells after a 24 hour incubation at 37°C using an anti-human IgG1-PE labeled detection antibody. Acquisition of data was performed on a FACS Canto II and analysis by FlowJo software. (C) ADCC induction of XGFR was determined with H322M tumor cells and NK92 effector cells. Cells were incubated at an effector/tumor cell ratio of 3:1 for 5 hours at the indicated concentrations of XGFR, XGFR2 and the parental control antibodies R7072 and GA201 in triplicate in two or more independent experiments. The xCELLigence technology and software was used for data analysis.

Figure 4. Inhibition of EGFR and IGF-1R receptor phosphorylation and 3D tumor cell proliferation in A549 cells. A549 NSCLC cells were incubated with XGFR or GA201/R1507 parental antibodies at concentrations between 200 and 0.0122 nM for 30 min in presence of 5nM IGF-1 or 10nM EGF. Phosphorylation of IGF-1R (A) and EGFR (B) was analyzed after cell lysis by P-EGFR (Tyr) or P-IGF-1R (Tyr) beads combined with a phosphoprotein detection reagent. The data were recorded in triplicate by the Luminox detection system. (C) Inhibition of cell viability by XGFR at concentrations between 8 and 0.0005 nM in comparison with GA201/R1507 was determined in a 3D cellular proliferation assay after 7 days of incubation.

Figure 5. Inhibition of EGFR and IGF-1R downstream signaling pathways by XGFR. LS174T colon carcinoma cells were incubated with XGFR, GA201 or R1507 parental antibodies at a concentration of 1000 nM in triplicates for 0.5, 6, 24 and 48 hours. Levels of phosphorylated IGF-1R (A), RAF1 (B) AKT (C) and ERK1 (D) in total cell lysates were determined by the Luminox assay technology. Mean levels of relative phosphorylation in comparison to IgG treated controls are depicted.
Figure 6. XGFR in vivo efficacy in human AsPC-1 luciferase pancreatic carcinoma mouse model. A, Bioluminescence signal of a representative example derived from AsPC-1 luc cells implanted orthotopically into the pancreas of SCID mice on day 7, 14, 21 and 27 after tumor inoculation. Upper panel: treatment with Omalizumab (control antibody), lower panel: treatment with XGFR at 20 mg/kg, i.p. once weekly. B, Quantification of mean bioluminescence intensity (BLI) in n=5 animals treated with Omalizumab (control antibody) or XGFR (20 mg/kg, i.p. once weekly) at indicated timepoints after orthotopic tumor cell inoculation.

Figure 7: XGFR in vivo efficacy in lung and colon carcinoma in vivo models. A, Kaplan-Meier plot of indicated treatment groups (n=10-15) in an orthotopic A549 NSCLC survival model in SCID beige mice. B, Kaplan-Meier plot of indicated treatment groups (n=10) in an intrasplenic LS174T colon carcinoma survival model in SCID beige mice. Treatment start was at day 7 after tumor inoculation in both models with weekly intraperitoneal administration of vehicle or test compounds.
**TABLES**

**Table 1.** Protein Purification yields of XGFR molecules compared to parental antibodies GA201 and R1507

<table>
<thead>
<tr>
<th>Transient HEK293 Expression</th>
<th>Purification Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecule</td>
<td>Protein A (mg/L)</td>
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<tr>
<td>XGFR</td>
<td>24.0</td>
</tr>
<tr>
<td>XGFR-DVD</td>
<td>1.5</td>
</tr>
<tr>
<td>XGFR2</td>
<td>13.3</td>
</tr>
<tr>
<td>GA201</td>
<td>26.4</td>
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<tr>
<td>R1507</td>
<td>32.5</td>
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**Table 2.** SPR analysis of the binding affinities of XGFR molecules to EGFR

<table>
<thead>
<tr>
<th>Binding to EGFR</th>
<th>K_D (nM)</th>
<th>K_a (1/Ms)</th>
<th>K_d (1/s)</th>
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<tr>
<td>Molecule</td>
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<tr>
<td>XGFR</td>
<td>4</td>
<td>8.20E+04</td>
<td>5.80E-04</td>
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<td>1.99E-04</td>
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<tr>
<td>XGFR2</td>
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<td>GA201</td>
<td>3</td>
<td>5.92E+04</td>
<td>1.91E-04</td>
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Table 3. SPR analysis of the binding affinities of XGFR molecules to IGF-1R

<table>
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<tr>
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<th>K_D (nM)</th>
<th>K_a (1/Ms)</th>
<th>K_d (1/s)</th>
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<td>-</td>
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Table 4. FACS Quantification of EGF and IGF-1 receptor density on tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGF receptors / cell</th>
<th>IGF-1 receptors / cell</th>
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<tbody>
<tr>
<td>AsPC-1</td>
<td>96645</td>
<td>15019</td>
</tr>
<tr>
<td>HT-29</td>
<td>18165</td>
<td>7287</td>
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<td>H322M</td>
<td>62480</td>
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<tr>
<td>A549</td>
<td>39695</td>
<td>43205</td>
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</tbody>
</table>
Figure 1

A

GA201

R1507

XGFR

XGFR2

XGFR-DVD

B

Reducing SDS-PAGE
Figure 2

A Analytical SEC

B CE-SDS (non-reduced, reduced)

C

<table>
<thead>
<tr>
<th>MS analysis</th>
<th>Homodimer (hole-hole)</th>
<th>Homodimer (knob-knob)</th>
<th>½ antibody</th>
<th>⅔ antibody</th>
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</thead>
<tbody>
<tr>
<td>XGFR</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
</tbody>
</table>
Figure 3

A

% IGF-1R Levels in Cell Lysate

B

Mean Fluorescence intensity

C

% Cytotoxicity

by guest on October 5, 2017 http://www.jbc.org/ Downloaded from
Figure 4

A

% IGF-1R Phosphorylation Inhibition

B

% EGFR Phosphorylation Inhibition

C

% Inhibition of Proliferation
Figure 5

A. Phosphorylated IGF-1R

B. Phosphorylated RAF1

C. Phosphorylated AKT

D. Phosphorylated ERK1

GA201
R1507
XGFR
Figure 6

A

AsPC1 Bioluminescence Imaging

Control Antibody

XGFR

Note: one representative mouse per treatment group

B

Mean Total BLI Signal [counts/s] ± SEM

Study Day

Control Antibody (20 mg/kg)

XGFR (20 mg/kg)
Figure 7

A

A549 NSCLC/SCID beige

B

LS174T SCID beige
A novel glycoengineered bispecific antibody format for targeted inhibition of EGFR and IGF-1R demonstrating unique molecular properties

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