Preclinical Assessment of AMG 596, a Bispecific T-cell Engager (BiTE) Immunotherapy Targeting the Tumor-specific Antigen EGFRvIII

Alexander Sternjak[1], Fei Lee[2], Oliver Thomas[1], Mercedesz Balazs[3], Joachim Wahl[1], Grit Lorenczewski[1], Ines Ullrich[4], Markus Muenz[4], Benno Rattel[1], Julie M. Bailis[2], and Matthias Friedrich[1]

ABSTRACT

AMG 596 is a bispecific T-cell engager (BiTE) immuno-oncology therapy in clinical development for treatment of glioblastoma multiforme (GBM), the most common primary brain tumor in adults with limited therapeutic options. AMG 596 is composed of two single-chain variable fragments that simultaneously bind to the tumor-specific antigen, EGFR variant III (EGFRvIII), on GBM cells and to CD3 on T cells, thereby activating T cells to proliferate and secrete cytotoxic substances that induce lysis of the bound tumor cell. T-cell–redirected lysis by AMG 596 is very potent; *in vitro* studies revealed EC_{50} values in the low picomolar range, and *in vivo* studies showed that AMG 596 treatment significantly increased the overall survival of mice bearing EGFRvIII-expressing orthotopic tumors. In addition, AMG 596 activity is highly specific; no AMG 596–induced T-cell activity can be observed in assays with EGFRvIII-negative GBM cells, and no signs of toxicity and activity were observed in cynomolgus monkeys, which lack expression of EGFRvIII on normal tissues. With EGFRvIII-expressing GBM cells, we showed shedding of EGFRvIII-containing membrane vesicles, followed by vesicle uptake and EGFRvIII cell surface presentation by EGFRvIII noncoding GBM cells. Cell membrane presentation of EGFRvIII following microvesicle transfer allows engagement by AMG 596, resulting in T-cell activation and T-cell–dependent lysis of GBM cells. Together, these data show a compelling preclinical efficacy and safety profile of AMG 596, supporting its development as a novel immunotherapy for treatment of GBM.

Introduction

Glioblastoma multiforme (GBM), or grade IV malignant glioma, is a highly aggressive malignant primary brain tumor and the most common primary brain tumor in adults, with an incidence of two to three new cases per 100,000 people per year in Europe and North America (1–3). The incidence of GBM has been increasing over the last 2 decades (3), and it now accounts for approximately 50% of all primary malignant central nervous system tumors in the United States (1). Patients diagnosed with GBM have extremely poor prognosis despite recent advances in treatment (4). Median survival for patients with GBM receiving standard-of-care therapy, surgery, and adjuvant chemoradiation is 15 to 16 months (5). Consequently, there is an urgent need to develop new therapeutic options that prolong patient survival.

EGFR variant III (EGFRvIII) is a tumor-associated antigen that is a promising therapeutic target because of its specific expression in tumors and its role in tumor cell growth (6). EGFRvIII is a deletion variant of EGFR and is expressed in up to one-third of all cases of GBM, but is not expressed on any normal tissues (6, 7). EGFRvIII has an extracellular domain truncation lacking EGFR exons 2 through 7, which abolishes EGF ligand binding and converts EGFRvIII into a constitutively active protein kinase that upregulates several pathways involved in tumor growth and metastasis (6). We previously developed an EGFRvIII-targeted antibody–drug conjugate, AMG 595, which was evaluated in a phase I study in patients with GBM. Despite evidence for antitumor activity (2/32 patients achieved a partial response and 15/32 patients demonstrated stable disease), further clinical development of AMG 595 was discontinued (8). Chimeric antigen receptor (CAR) T cells binding EGFRvIII (EGFRvIII CAR T cell) have also been evaluated in patients with GBM (9). EGFRvIII CAR T cells demonstrated evidence for on-target activity, including trafficking to and expansion of CAR T cells at tumor sites and direct killing of EGFRvIII-expressing tumor cells, with stable disease achieved by one in 10 patients treated (9). Together, these clinical studies highlight the promise of targeting EGFRvIII and the potential to use immunotherapy for treatment of GBM.

Bispecific T-cell engager (BiTE) molecules (Amgen Inc.) are composed of two single-chain variable fragments (scFv) connected by a short peptide linker. One scFv is derived from an antibody specific to a tumor-associated antigen on tumor cells, and the other scFv binds to CD3 expressed on T cells. Concurrent binding of a BiTE molecule to tumor cells and T cells leads to T-cell activation, T-cell expansion, and redirected T-cell lysis of tumor cells (ref. 10; Supplementary Fig. S1). The first BiTE molecule, blinatumomab, targets CD19 and is the only approved T-cell–bispecific molecule on the global market. Blinatumomab is approved for treatment of B-cell acute lymphoblastic leukemia and has demonstrated clinical activity in non-Hodgkin lymphoma (11, 12). The BiTE molecule, AMG 212, which targets prostate-specific membrane antigen, has shown early evidence for clinical activity in a solid tumor setting (13). These experiences led us to develop AMG 596, a BiTE molecule targeting EGFRvIII, as a novel, targeted immunotherapy for GBM.

[1]Translational Safety & Bioanalytical Sciences, Amgen Research (Munich) GmbH, Munich, Germany. [2]Department of Oncology Research, Amgen Research, Amgen Inc., South San Francisco, California. [3]Translational Safety & Bioanalytical Sciences, Amgen Research, Amgen Inc., South San Francisco, California. [4]Therapeutic Discovery, Amgen Research (Munich) GmbH, Munich, Germany.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Alexander Sternjak, Translational Safety & Bioanalytical Sciences, Amgen Research (Munich) GmbH, Munich 81477, Germany. Phone: 4989-8952-77515; Fax: 4989-8952-77235; E-mail: sternjak@amgen.com

Mol Cancer Ther 2021;20:925–33

doi: 10.1158/1535-7163.MCT-20-0508

AMG 596 is composed of an EGFRvIII scFv derived from AMG 595 connected to an scFv specific for the CD3 molecule expressed on T cells (8, 14). AMG 596 is a fully human BiTE molecule that cross-reacts with cynomolgus monkey CD3. AMG 596 demonstrated potent, specific cell killing activity against EGFRvIII-positive GBM cells *in vitro* and showed remarkable antitumor activity in an orthotopic xenograft tumor model in mice. In cynomolgus monkeys, AMG 596 was well-tolerated at doses up to 450 μg/kg/day, with no evidence of T-cell activity, consistent with the tumor-specific expression of EGFRvIII. These findings supported the development of AMG 596 as an immuno-oncology therapy for patients with GBM with EGFRvIII-positive tumors.

Materials and Methods

Generation of AMG 596

AMG 596 is a bispecific construct composed of an EGFRvIII-binding scFv at the amino terminus and a CD3-binding scFv at the carboxyl terminus. The binding domains of AMG 596 are cross-reactive to human and cynomolgus monkey targets. However, because EGFRvIII is a tumor-specific antigen, it is not naturally expressed in cynomolgus monkeys. A putative cynomolgus monkey EGFRvIII sequence was derived from comparison of the cynomolgus monkey and human EGFR cDNA sequences and application of the human EGFRvIII mutation pattern to the cynomolgus monkey sequence (Supplementary Fig. S2). AMG 596 was expressed in Chinese hamster ovary (CHO) cells (ATCC) and harvested from the supernatant by a two-step purification process consisting of immobilized metal affinity chromatography followed by size exclusion chromatography, as reported for other BiTE molecules (14, 15). The AMG 596 sequence is disclosed in sequence ID No. 160 in patent No. US 10,519,241 B2; an additional 6× His Tag has been added.

Cell culture

Tumor cell lines, U-251 MG and EGFRvIII-expressing U-251 MG (U-251 MG/EGFRvIII), were described previously (16). Both cell lines were cultured in DMEM (Gibco) containing 10% FBS (Invitrogen) and 100 U/mL penicillin/streptomycin (Gibco). Tumor cell lines, U-87 MG/EGFRvIII and GOS-3/EGFRvIII, were generated by transduction of U-87 MG tumor cells (ATCC) and GOS-3 [German Collection of Microorganisms and Cell Culture (DSMZ)] with human EGFRvIII cDNA, and were cultured in RPMI1640 (Biochrom) and DMEM containing 10% FBS, 100 U/mL penicillin/streptomycin, and 5 μg/mL Blasticidin (Gibco). The tumor cell line, DK-MG (DSMZ), was cultured in RPMI1640 containing 10% FBS, 100 U/mL penicillin/streptomycin, and 1 × nonessential amino acids (NEAA, Biochrom). CHO cells were transduced with wild-type EGFR (HER1), HER2, HER3, HER4, and human and cynomolgus monkey EGFRvIII. CHO cells were cultured in DMEM containing 10% FBS, 10 mmol/L sodium hypoxanthine, 1.6 mmol/L thymidine (1 × HT Supplement, Gibco), and 1 × NEAA (Biochrom). Transduced CHO cells were cultured in DMEM containing 10% FBS, 2 mmol/L L-Glutamine (Biochrom), 100 U/mL penicillin/streptomycin, and 1 × NEAA. T-cell lines, HPB-ALL (DSMZ) and LnPx4119 (described previously; ref. 15), were cultured in RPMI1640 containing 10% FBS and 100 U/mL penicillin/streptomycin. All cells were cultured at 37°C in a 5% CO_2 chamber.

In vitro assays

On-cell binding of AMG 596 was assessed on CD3-expressing human and monkey (*Macaca mulatta*) T-cell lines (HPB-ALL and LnPx4119) and CHO cells expressing human EGFR/HER1, HER2, HER3, HER4, and EGFRvIII, and cynomolgus monkey EGFRvIII by flow cytometry. Cells were incubated with 5 μg/mL of AMG 596 or the Mec14 BiTE molecule specific for the herbicide mecoprop (15), followed by incubations with a biotin-conjugated secondary antibody (Dianova) and APC-conjugated Streptavidin [Becton Dickinson (BD)].

T-cell–dependent cellular cytotoxicity (TDCC) assays were assessed by flow cytometry using human or cynomolgus monkey peripheral blood mononuclear cells (PBMCs) as effector cells and U-251 MG, U-251 MG/EGFRvIII, U-87 MG/EGFRvIII, GOS-3/EGFRvIII, DK-MG, and CHO cells transduced with cynomolgus monkey EGFRvIII (CHO/cynoEGFRvIII) as target cells. PBMC were isolated from human ("Institut für Klinische Transfusionsmedizin und Immungenetik Ulm," Ulm, Germany) and cynomolgus monkey blood (WIL Research) by Ficoll (Sigma-Aldrich) density gradient centrifugation. Target cells were labeled with Vybrant DiO (Invitrogen) and cocultured with effector cells at effector to target cell (E:T) ratios of 10:1 and increasing concentrations of AMG 596 in RPMI1640, containing 10% FBS and 100 U/mL penicillin/streptomycin, at 37°C in a 5% CO_2 chamber. Target cell lysis was analyzed by flow cytometry as loss of cell membrane integrity (uptake of propidium iodide) after 48 (human assays) and 72 hours (cynomolgus monkey assays). T-cell activation was evaluated by flow cytometry after 48 hours. T cells were identified by antibodies against CD4 and CD8 and their activation status was determined with an anti-CD25 antibody (antibodies from BD). T-cell activation and redirected lysis were analyzed on a FACSCanto II Flow Cytometer (BD) and data were evaluated using FACS Diva Software (BD) and GraphPad Prism (GraphPad Software). Cytokine release was monitored in supernatants of TDCC assays using a Cytometric Bead Array (BD) according to the manufacturer's protocol.

Microvesicle assays

U-251 MG/EGFRvIII cells were cultured in DMEM with 10% exosome-depleted FBS (Gibco). Medium was centrifuged at 500 × *g* for 5 minutes to remove dead cells and cell debris, followed by filtration through an 0.8-μm filter (Millipore). Microvesicles were pelleted by ultracentrifugation (Optima L-100K Ultracentrifuge, Beckman Coulter) at 100,000 × *g* for 80 minutes at 8°C. The pellet was washed with cold PBS and collected by ultracentrifugation at 100,000 × *g* for 8 minutes at 8°C. Purified microvesicles were suspended in either PBS or fresh cell culture medium. The protein concentration of the microvesicle preparation was determined by BCA Protein Assay (Pierce).

U-251 MG or U-251 MG/EGFRvIII cells were lysed in RIPA Buffer (Thermo Fisher Scientific) with 1× Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Protein (15 μg) from microvesicles or from RIPA lysates of U-251 MG or U-251 MG/EGFRvIII cells was resolved by NuPAGE Bis-Tris Mini Gel 4% to 12% (Thermo Fisher Scientific) and transferred to a nitrocellulose membrane. Immunoblotting was carried out using iBind Western System and iBind FD Solution Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Primary antibodies against EGFRvIII (Amgen) or β-actin (Cell Signaling Technology) were detected with IRDye-conjugated secondary antibodies (LI-COR). Protein bands were visualized using LI-COR Odyssey CLx Imaging System (Li-COR).

U-251 MG cells were incubated with various amounts of microvesicles (0–200 μg) for 24 hours. Cell surface EGFRvIII levels were detected by incubation with AMG 596, followed by a secondary fluorescently labeled antibody (Miltenyi Biotec), and analyzed by flow cytometry using an LSR Fortessa and FlowJo Software (BD).

U-251 MG cells were incubated with various amounts of microvesicles (0–200 μg) for 24 hours. Human pan-T cells (AllCells) were added at an E:T ratio of 10:1 with a dose range of AMG 596. Microvesicles were added simultaneously with T cells and AMG 596 to maintain consistent microvesicle concentrations. After 48 hours, T-cell activation was analyzed by flow cytometry, and tumor cell killing was assessed by the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

Antibodies used for analysis of T-cell activation were directed against CD3, CD4, and CD25 (BD), and CD8 (BioLegend). Cell viability was assessed with a Fixable Viability Dye (eBioscience).

Mouse xenograft studies

All experiments were performed according to the German Animal Protection Law with permission from the responsible local authorities. Animals were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*, 8th edition (17). Antitumor activity was evaluated in xenograft tumor models in 6-week-old female NOD.CB17-*Prkdc*^{scid}/J mice (NOD/SCID; Charles River Laboratories). Before tumor cell injection, mice were sublethally irradiated (2 Gy) to facilitate and ensure tumor cell growth and human T-cell engraftment. In addition, a single intravenous bolus injection of anti-asialo GM1 rabbit antibody (Biozol) was administered 1 day before the intraperitoneal injection of human T cells (2×10^7 T cells/mouse) to deplete murine natural killer cells. T cells were originated from a single healthy donor and were activated and expanded using a T-cell Activation/Expansion Kit (Miltenyi Biotec).

For the orthotopic tumor model, mice were anesthetized with a Ketamine/Xylazine Mixture (100/8 mg/kg, i.p. injection, CP-Pharma) and received Rimadyl (5 mg/kg, s.c., Pfizer) for analgesia. Mice were fixed in a Stereotactic Device (Kopf Instruments) and the skull was trepanned with a 22 G needle at the injection site (3.5 mm anterior of the interaural line and 2 mm lateral of the midline). A Microliter Syringe (Hamilton) adjusted to the stereotactic device was used to inject U-87 MG/EGFRvIII tumor cells (1×10^5 cells/mouse) into the right cerebral hemisphere. T cells were injected at day 4, and AMG 596, the vehicle control, and the Mec14 BiTE molecule were administered by intravenous bolus into the lateral tail vein once daily for 16 consecutive days starting on day 8. Statistical analysis of survival was performed using the log-rank (Mantel–Cox) test.

For the subcutaneous tumor model, U-251 MG/EGFRvIII cells (1×10^7 cells/mouse mixed with Matrigel, R&D Systems) were injected subcutaneously into the right dorsal flank. T cells were injected at day 9 when tumors had reached a volume of approximately 200 mm^3. At day 11, AMG 596 and vehicle were administered by intravenous bolus into the lateral tail vein once daily for 11 consecutive days. Tumor growth was monitored by external caliper measurements, and tumor volumes were calculated using a standard hemi ellipsoid formula: [length (mm) × width (mm)^2]/2.

Tumor histology and IHC analysis of murine brain sections

Whole mouse brains from the orthotopic tumor model were embedded in Tissue-Tek OCT Compound (Hartenstein) and cut to 3- to 5-μm-thick sections. Sections were transferred onto slides (Hartenstein), air dried, and fixed in cold Acetone (Sigma-Aldrich). For histologic evaluation, brain sections were stained with hematoxylin (Vector Laboratories) and eosin (Sigma-Aldrich). Evaluation of EGFRvIII and CD3 expression included blocking of endogenous peroxidase activity by a Peroxidase Blocking Reagent (Dako North America), and incubation with 10 μg/mL of murine-antihuman EGFRvIII (NewEast Biosciences) and murine-antihuman CD3 (Sigma-Aldrich) or a murine IgG isotype control antibody (Bio-Rad). Antibody binding was detected by incubation with a peroxidase-labeled polymer conjugated to goat-anti mouse IgG followed by the incubation with a DAB + Peroxidase Substrate (both Dako). Slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylol, and covered with Roti HistoKitt II (Carl Roth GmbH). Tissue slides were recorded with a LM2500 light microscope using a DFC295 Camera (Leica Microsystem).

Cynomolgus monkey study

Male cynomolgus monkeys were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*, 8th edition (17). Animals were housed individually at an indoor, Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility, and all research protocols were approved by the Institutional Animal Care and Use Committee. Before continuous intravenous infusion of AMG 596, animals were implanted with a vascular access port/catheter into the femoral vein. The catheter was attached to a battery-powered Infusion Pump (SIMS Deltec) located in the pocket of a jacket placed on each animal, and dosing solutions were administered via continuous intravenous infusion for 7 days. Throughout the study, animals were monitored for general health/mortality and moribundity twice daily. Both before and during dosing, body temperature, body weight, and food consumption were monitored, and hematology, coagulation, serum chemistry, lymphocyte subtypes, and serum cytokine levels were analyzed. At study end, animals were necropsied and macroscopically assessed for noticeable changes. Tissues from selected organs were prepared for subsequent histopathologic examinations.

Key resources of "Materials and Methods," their sources, and identifiers can be found in Supplementary Table S1.

Results

The BiTE immunotherapy AMG 596 binds EGFRvIII and CD3

AMG 596 is a targeted immunotherapy that binds to human EGFRvIII on tumor cells and human CD3 on T cells. It is composed of an EGFRvIII-binding scFv located at the amino terminus that is connected via a short peptide linker to the carboxy terminal CD3-binding scFv (**Fig. 1A**). AMG 596 is a fully human BiTE molecule that was designed to cross-react with cynomolgus monkey CD3 and with a putative cynomolgus monkey EGFRvIII. Flow cytometry analysis confirmed AMG 596 binding to CHO cells that stably expressed either human or cynomolgus monkey EGFRvIII, but not to CHO cells that did not express the target (**Fig. 1B**). AMG 596 binding was specific for EGFRvIII as no binding was observed to CHO cells engineered to express either wild-type EGFR (HER1) or the EGFR family members HER2, HER3, and HER4 (**Fig. 1B**). An anti-EGFR BiTE molecule, based on the sequence of an anti-EGFR antibody binding to both wild-type EGFR and EGFRvIII (15), was used as a positive control. No binding to EGFRvIII was observed with the nontargeting Mec14 BiTE molecule. AMG 596 also demonstrated specific binding to the CD3-expressing human and monkey T-cell lines, HPB-ALL and LnPx4119, by flow cytometry (**Fig. 1C**).

AMG 596 induces potent cytotoxic activity against EGFRvIII-expressing target cells

BiTE immunotherapy acts by directly connecting target cells to T cells, leading to T-cell activation, cytokine release, and redirected lysis. These pharmacologic effects of AMG 596 were evaluated in human

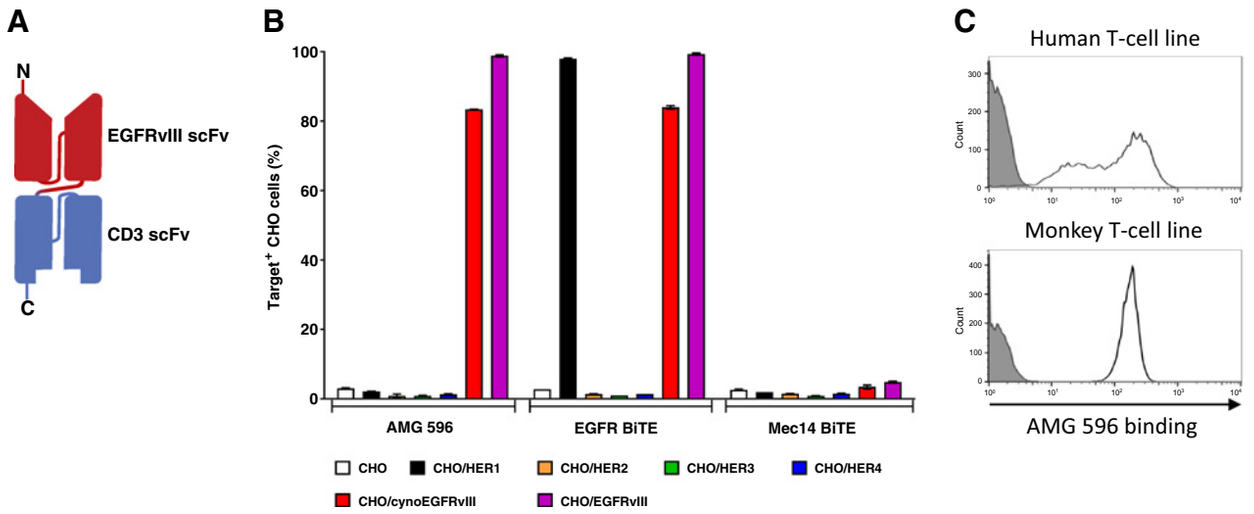

Figure 1.

AMG 596 domain arrangement and binding specificity to EGFRvIII and CD3. **A,** Schematic presentation of the domain arrangement of AMG 596. C, carboxyl terminus; N, amino terminus. **B,** Flow cytometry was used to assess AMG 596 on-cell binding to CHO cells alone, CHO cells stably expressing EGFR/HER1 (CHO/HER1), HER2 (CHO/HER2), HER3 (CHO/HER3), or HER4 (CHO/HER4), and CHO cells engineered to express human EGFRvIII (CHO/EGFRvIII) or CHO/cynoEGFRvIII. EGFR BiTE molecule and the Mec14 BiTE molecule were used as positive and negative controls. **C,** AMG 596 on-cell binding to CD3 (open histogram) on the human T-cell line, HPB-ALL (top), and the monkey T-cell line, LnPx4119 (bottom), was evaluated by flow cytometry. Solid histograms show background staining with AMG 596 detection reagents.

and cynomolgus monkey cell–based assay systems to investigate whether cynomolgus monkey could serve as a relevant species for pharmacologic and toxicologic assessments.

AMG 596–induced activation of human and cynomolgus monkey T cells was demonstrated in cocultures containing U-251 MG/EGFRvIII cells and human or cynomolgus monkey PBMC. Flow cytometric analysis revealed that AMG 596 treatment led to dose-dependent upregulation of the cell surface T-cell activation marker, CD25, in both species. No T-cell activation was detected when U-251 MG cells that did not express EGFRvIII were treated with AMG 596, demonstrating the requirement of EGFRvIII expression for AMG 596–mediated T-cell activation (**Fig. 2A** and **B**).

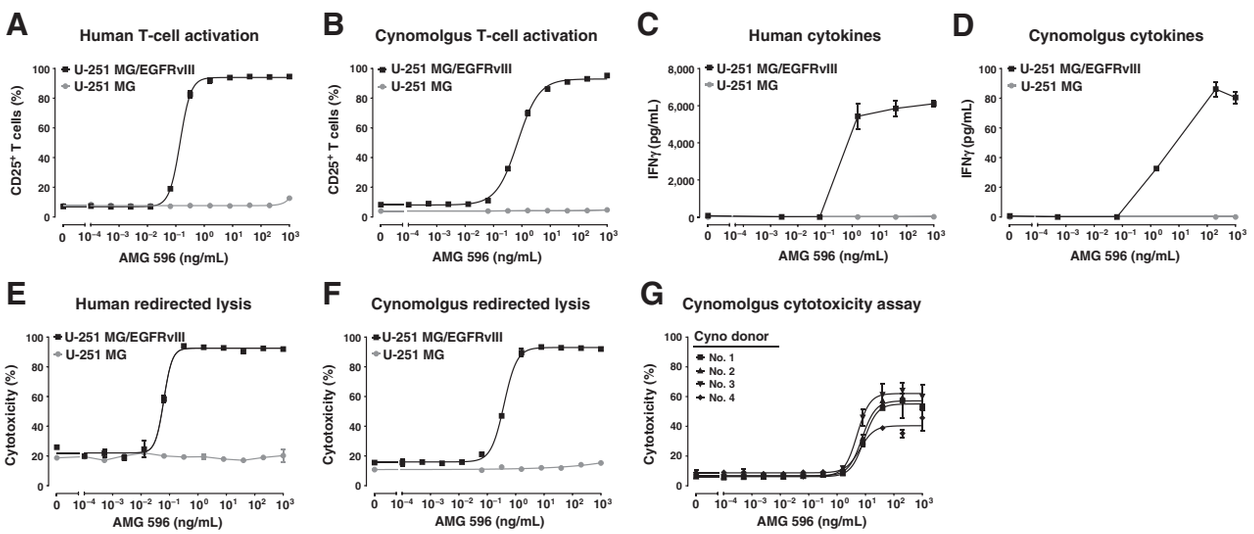

Figure 2.

AMG 596 activity in cytotoxicity assays with human and cynomolgus monkey effector cells. U-251 MG/EGFRvIII and U-251 MG tumor cells were cocultured with human (**A** and **C**) and cynomolgus monkey (**B** and **D**) PBMC at E:T ratios of 10:1 and AMG 596 for 48 hours. Upregulation of CD25 on T cells and IFNγ in assay supernatants was analyzed by flow cytometry. U-251 MG/EGFRvIII and U-251 MG tumor cells were cocultured with human (**E**) and cynomolgus monkey (**F**) PBMC at E:T ratios of 10:1 and AMG 596 for 48 (human) and 72 hours (cynomolgus monkey). Target cell lysis was analyzed by flow cytometry. **G,** CHO/cynoEGFRvIII cells were cocultured with cynomolgus monkey PBMC at E:T ratios of 10:1 and AMG 596 for 72 hours. Redirected lysis was monitored via flow cytometry. Data points represent the mean of duplicate measurements. Error bars indicate the SEM.

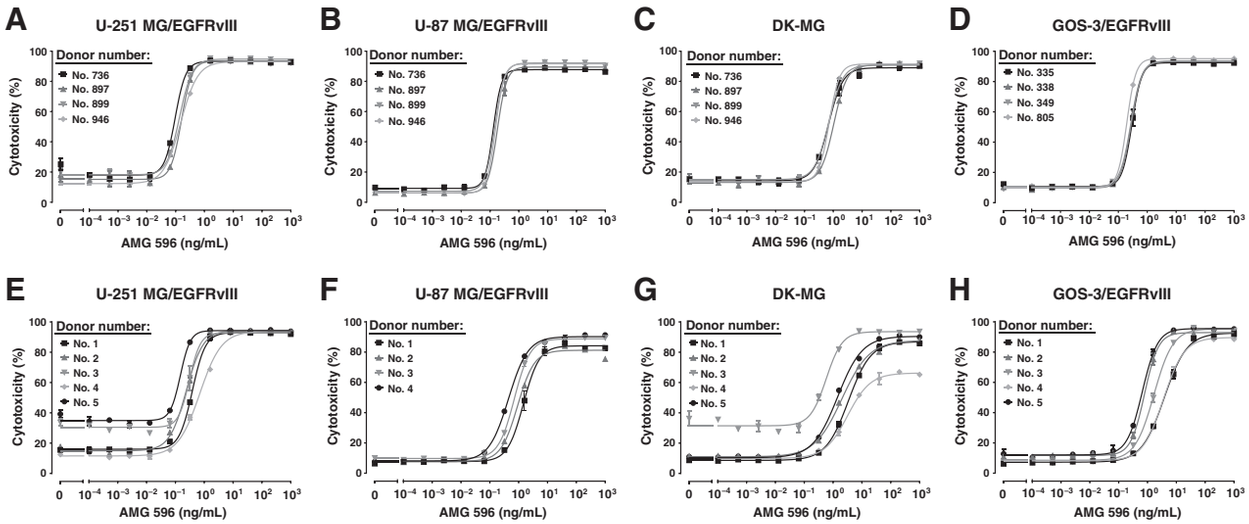

Figure 3.

AMG 596–mediated lysis of various EGFRvIII-expressing GBM cells by human or cynomolgus monkey effector cells. EGFRvIII-expressing GBM cells (U-251 MG/EGFRvIII, U-87 MG/EGFRvIII, DK-MG, and GOS-3/EGFRvIII) were cocultured with human (**A–D**) and cynomolgus monkey PBMC (**E–H**) at E:T ratios of 10:1 and increasing AMG 596 concentrations for 48 (human) or 72 hours (cynomolgus monkey). Redirected lysis was evaluated by flow cytometry. Data points represent the mean of duplicate measurements. Error bars indicate SEM.

The BiTE molecule mechanism of action is associated with dose-dependent release of cytokines (14, 18). Therefore, cytokines were analyzed in supernatants from AMG 596 cytotoxicity assays containing U-251 MG/EGFRvIII cells and human or cynomolgus monkey PBMC. AMG 596 induced a dose-dependent release of cytokines by human and cynomolgus monkey effector cells as exemplified by IFNγ (**Fig. 2C** and **D**). With effector cells from both species, IFNγ secretion was observed at similar AMG 596 concentrations and was strictly dependent on the presence of EGFRvIII-positive target cells. No IFNγ was observed in assays with U-251 MG cells that did not express EGFRvIII, confirming that AMG 596 activity requires engagement of both EGFRvIII and CD3 on T cells.

Specificity of redirected lysis by human and cynomolgus monkey effector cells was confirmed in AMG 596 cytotoxicity assays. While EGFRvIII-positive U-251 MG cells were efficiently lysed, AMG 596 treatment did not affect the viability of target-negative U-251 MG cells (**Fig. 2E** and **F**). Moreover, cytotoxicity data obtained with different EGFRvIII-expressing GBM cell lines (U-251 MG/EGFRvIII, GOS-3/EGFRvIII, U-87 MG/EGFRvIII, and the EGFRvIII endogenously expressing cell line DK-MG) revealed that T-cell–mediated lysis was comparable between the two species (**Fig. 3**), although EC_{50} values calculated from dose–response curves with human T cells were somewhat lower (∼5-fold; Supplementary Table S2), suggesting that AMG 596 is slightly more potent in human assay systems. Finally, AMG 596 cross-reactivity to cynomolgus monkey was tested in cytotoxicity assays with cynomolgus monkey PBMC and CHO/cynoEGFRvIII cells. These assays confirmed the capability of AMG 596 to effectively engage cynomolgus monkey EGFRvIII and CD3 to promote target cell lysis (**Fig. 2G**), and further supported the use of the cynomolgus monkey as a relevant species for safety testing.

Uptake of EGFRvIII microvesicles enables AMG 596–mediated redirected lysis

The EGFRvIII antigen is reported to be transferred between tumor cells through microvesicles derived from the plasma membrane (19). To test whether this occurs in our GBM cell line models, we isolated microvesicles from U-251 MG/EGFRvIII tumor cells and used Western blot analysis to confirm the presence of EGFRvIII protein in the microvesicle preparation (**Fig. 4A**). We then incubated U-251 MG tumor cells with increasing amounts of isolated EGFRvIII-positive microvesicles and evaluated EGFRvIII transfer to the cell surface of the U-251 MG cells by flow cytometry. Increasing levels of cell surface EGFRvIII were detected after incubation with increasing amounts of EGFRvIII-positive microvesicles (**Fig. 4B**), thus confirming transfer of EGFRvIII to the U-251 MG cells and expression on the cell membrane. To test whether U-251 MG cells could be engaged by AMG 596 after EGFRvIII microvesicle uptake to drive T-cell activation and redirected lysis, we preincubated U-251 MG cells with increasing amounts of EGFRvIII microvesicles and, thereafter, cocultured them with human T cells and AMG 596 for 48 hours. AMG 596 effectively induced upregulation of the T-cell activation marker, CD25, on both $CD4^+$ and $CD8^+$ T cells (**Fig. 4C**) and mediated redirected lysis of microvesicle EGFRvIII-transferred U-251 MG cells (**Fig. 4D**). Both T-cell activation and redirected lysis increased in a dose-dependent manner when higher concentrations of AMG 596 were applied or when U-251 MG cells with higher EGFRvIII levels were used in the assay. Together, these data demonstrate that EGFRvIII can be transferred by microvesicle from EGFRvIII-positive cancer cells to EGFRvIII-negative cancer cells, thereby converting target-negative cells to target-positive cells that can be effectively engaged by AMG 596 immunotherapy.

In vivo antitumor activity of AMG 596

The antitumor activity of AMG 596 was evaluated in an orthotopic xenograft brain tumor model in NOD/SCID mice. U-87 MG/EGFRvIII cells (AMG 596–induced redirected lysis, T-cell activation, and cytokine production were demonstrated in assays with U-87 MG/EGFRvIII cells; Supplementary Fig. S3) were implanted intracerebrally and tumors were allowed to grow. Human T cells were

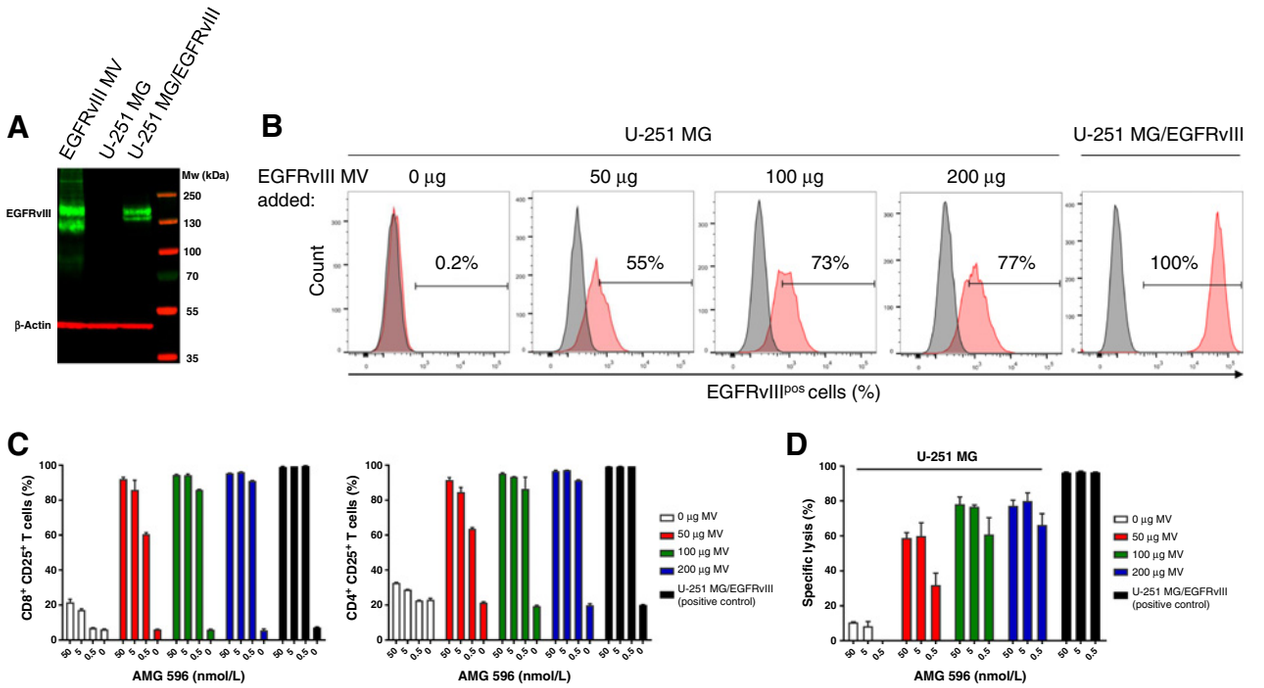

Figure 4.

Uptake of EGFRvIII-containing vesicles by target-negative tumor cells and their AMG 596–mediated redirected lysis. **A,** Western blot analysis of microvesicles (MV) isolated by ultracentrifugation from U-251 MG/EGFRvIII tumor cells (EGFRvIII MV) along with U-251 MG and U-251 MG/EGFRvIII tumor cells. **B,** U-251 MG cells were incubated with indicated amounts of EGFRvIII-containing microvesicles for 24 hours. Transfer of EGFRvIII to U-251 MG cells was analyzed by flow cytometry (percentages of EGFRvIII^{positive} cells; red histograms) using AMG 596 as a detection tool. EGFRvIII-positive U-251 MG/EGFRvIII cells were used as positive control. **C** and **D,** U-251 MG cells were preincubated with indicated amounts of EGFRvIII-containing vesicles and cocultured with T cells and different concentrations of AMG 596. After 48 hours of coculture, AMG 596–induced upregulation of CD25 on CD4$^+$ and CD8$^+$ T cells was analyzed by flow cytometry (**C**) and redirected lysis of U-251 MG cells that had taken up membrane EGFRvIII was assessed by a cell viability assay (**D**).

injected intraperitoneally once before initiation of BiTE molecule treatment. Animals were treated with AMG 596 (0.5 or 0.1 mg/kg), the Mec14 BiTE molecule (0.5 mg/kg, negative control), or vehicle only by daily intravenous bolus injection for 16 consecutive days starting on day 8 after the tumor cells were implanted. Mice treated with AMG 596 showed significantly prolonged survival compared with mice treated with either the Mec14 BiTE molecule or vehicle only ($P < 0.001$; **Fig. 5A**). At the end of the study (day 44), eight of 11 and 4 of 10 animals treated with 0.5 and 0.1 mg/kg AMG 596, respectively, were alive without macroscopic brain tumors at necropsy. In contrast, all mice treated with either the Mec14 BiTE molecule or control vehicle did not survive longer than 24 days after study start. These findings were confirmed by IHC and hematoxylin and eosin (H&E) staining of brain sections (**Fig. 5B**). No visible tumors were observed in AMG 596–treated animals, while large EGFRvIII-positive tumors were observed in vehicle-treated mice. IHC analysis of human T cells in brains of vehicle- and AMG 596–treated animals indicated T-cell infiltration, and possibly a trend toward increased numbers of T cells after AMG 596 treatment (Supplementary Fig. S4).

Furthermore, AMG 596 antitumor activity was also assessed in NOD/SCID mice bearing established subcutaneous GBM tumors. U-251 MG/EGFRvIII tumor cells were subcutaneously injected into NOD/SCID mice and grown to tumor volumes of approximately 200 mm^3. Then, human T cells were injected intraperitoneally once, followed by daily intravenous bolus injection of vehicle and AMG 596 (0.5 mg/kg) for 11 consecutive days. AMG 596 induced significant antitumor activity of U-251 MG/EGFRvIII tumors, while a steady increase in tumor volume was observed for vehicle-treated mice until day 22 (averaged tumor volume of 41 vs. 568 mm^3 for treatment and vehicle group; **Fig. 6**). These data again demonstrate the high antitumor activity of AMG 596 as monotherapy against established GBM tumors in mouse models.

AMG 596 safety assessment in cynomolgus monkey

An exploratory toxicology study was performed to evaluate EGFRvIII-independent effects that might be attributable to AMG 596 binding to only CD3 on T cells. A 7-day exploratory dose-range–finding study was conducted in which three male cynomolgus monkeys per group were administered vehicle or AMG 596 at doses of 15, 150, or 450 µg/kg/day via continuous intravenous infusion at dose volumes of 10 mL/kg/day. AMG 596 administration was very well-tolerated. Even at the highest dose with a steady-state serum concentration of 355 ng/mL, no clinical signs of toxicity or changes in body temperature or body weight were observed. Strikingly, the serum level of 355 ng/mL was about 15- and 197-fold higher than the effective concentration for 90% response (EC_{90}) values required for redirected lysis in AMG 596 TDCC assays with cynomolgus monkey (EC_{90} value, 24 ng/mL) and human PBMC (EC_{90} value, 1.8 ng/mL). In addition, levels of serum cytokines (MCP-1, IL6, IFNγ, TNFα, and IL2), usually an indicator for BiTE molecule activity *in vivo*, were not detected during the study. Moreover, clinical pathology parameters indicative of an acute phase response, such as C-reactive protein, did not differ between AMG

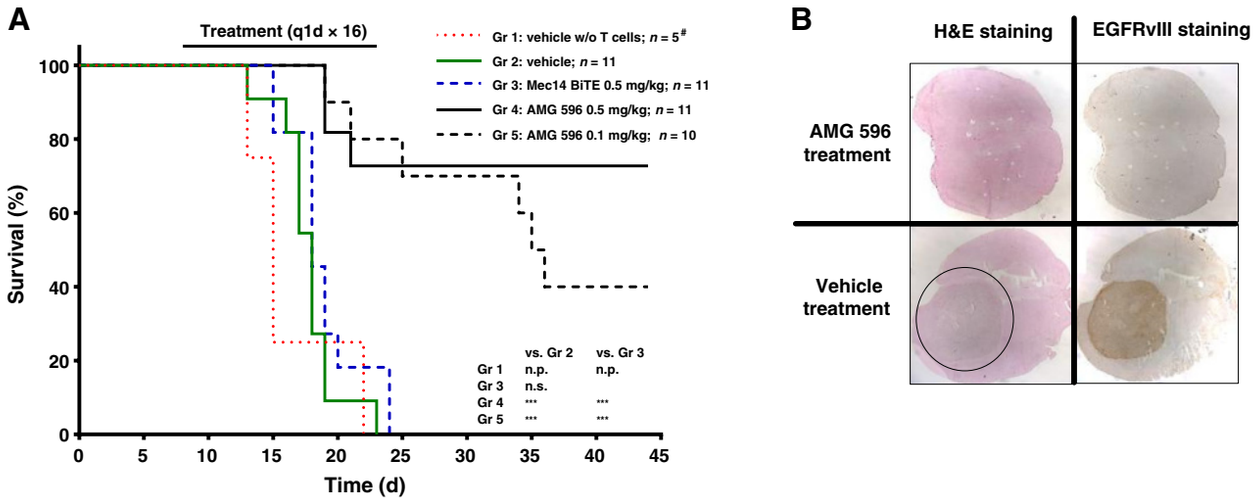

Figure 5.

AMG 596 treatment prolonged survival of mice bearing U-87 MG/EGFRvIII tumors. **A,** U-87 MG/EGFRvIII cells were injected into the right cerebral hemisphere of female NOD/SCID mice and human T cells were injected into the peritoneal cavity (except group 1) before treatment start at day 8. Control vehicle (groups 1 and 2), Mec14 BiTE molecule (group 3), and AMG 596 (groups 4 and 5) were administered by daily intravenous bolus injection for 16 consecutive days (every day ×16). **B,** Brain sections were prepared from vehicle- (group 2, at study day 17) and AMG 596–treated mice (group 4, at study day 44) and analyzed for tumor histology by H&E staining and EGFRvIII expression by IHC. Images were taken with a Leica LM2500 microscope (12.5× magnification). Tumor tissue, indicated by the region with the black circle in the bottom left panel, contained EGFRvIII-expressing cells (bottom right panel). Gr, group; *n*, number of animals in group; n.p., not performed; n.s., not significant. Asterisks denote statistically significant differences (log-rank test; ***, $P < 0.001$) between AMG 596–treated groups and control vehicle group 2 or the Mec14 BiTE molecule–treated group 3. #, one animal in group 1 died before treatment start and was excluded from analysis.

596- and vehicle-treated animals. Lack of AMG 596–mediated toxicity or activity was accompanied by the absence of histopathologic findings. This study clearly highlights the need for expression of both the target antigen and CD3 to obtain BiTE activity *in vivo*.

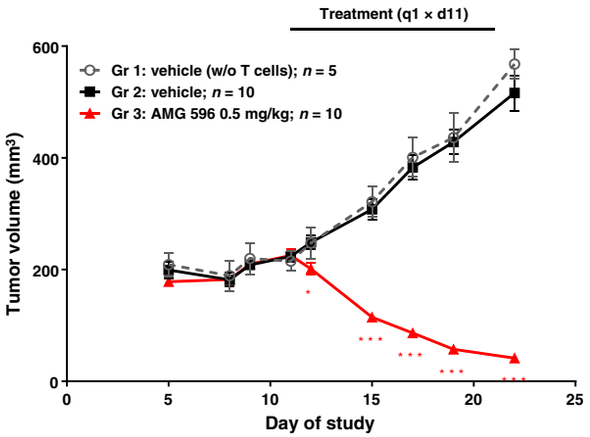

Figure 6.

AMG 596 treatment induced the regression of established subcutaneous U-251 MG/EGFRvIII tumors in mice. U-251 MG/EGFRvIII cells were subcutaneously injected into the right dorsal flank of NOD/SCID mice and human T cells were injected into the peritoneal cavity [except group (Gr) 1] before treatment start at day 11. Control vehicle (groups 1 and 2) and AMG 596 (group 3) were administered by daily intravenous bolus injection for 11 consecutive days (every day ×11). Tumor volumes were determined using electronic caliper measurements. Group mean tumor volumes (mm^3) ± SEM are shown. Asterisks in the figure denote statistically significant differences (one-way ANOVA; *, $P < 0.05$; ***, $P < 0.001$) between vehicle- (group 2) and AMG 596–treated group; *n*, number of animals.

Discussion

BiTE molecules are a clinically validated modality. The anti-CD19/CD3 BiTE molecule, blinatumomab, was approved by the FDA more than 5 years ago, and since that time several other BiTE molecules have demonstrated clinical activity, including in solid tumors (20). EGFRvIII is a promising immunotherapeutic target for the treatment of GBM owing to its specific expression on tumor cells. As with other BiTE molecules (14, 18, 21), nonclinical characterization of AMG 596 included analysis of *in vitro* and *in vivo* properties, as well as toxicologic evaluation in cynomolgus monkeys. Because EGFRvIII expression has not been reported in the cynomolgus monkey, AMG 596 was designed to bind to both human and a putative cynomolgus monkey EGFRvIII protein created by applying the human EGFRvIII mutation pattern to the highly homologous cynomolgus monkey EGFR sequence. AMG 596 possesses almost identical equilibrium KDs for human and cynomolgus monkey antigens (single-digit nmol/L range) with exclusive binding to EGFRvIII and no cross-reactivity to wild-type EGFR or other members of the EGFR family. Most importantly, *in vitro* human and cynomolgus monkey T-cell responses were comparable in terms of AMG 596–induced T-cell activation, cytokine secretion, and the magnitude of redirected lysis, which was shown to be similarly induced by $CD4^+$ and $CD8^+$ T cells (Supplementary Fig. S5). Slight differences were observed in EC_{50} values for redirected lysis (~5-fold lower in assays with human T cells), which could reflect variability in T cells from different donors, as well as differences between the two species. Similar findings were reported for other BiTE molecules and were considered when modeling human exposures based on toxicology and toxicokinetic data from cynomolgus monkey studies (14). In summary, *in vitro* data support the use of cynomolgus monkeys as a pharmacologically relevant species for toxicology testing. However, the absence of EGFRvIII expression on healthy cynomolgus monkey tissues indicated that toxicology studies

with AMG 596 would investigate only EGFRvIII-independent effects attributable to engagement of CD3 on T cells alone. This mimics the clinical situation in which EGFRvIII is reported to be expressed exclusively on tumors, but not on normal tissues (22–25). In fact, the EGFRvIII exploratory toxicology study showed that, even at high doses, AMG 596 did not induce any signs of activity or toxicity, demonstrating that BiTE molecule activity is not attributable solely to CD3 binding on T cells, but requires the additional concurrent binding of a tumor-associated antigen, in this case EGFRvIII. In addition, the absence of toxicity at high AMG 596 serum concentrations in this study suggested that there was no potential cross-reactivity to EGFR, which has widespread expression on healthy tissues that caused massive cytokine release leading to intolerability in cynomolgus monkeys treated with a cross-reactive EGFR BiTE molecule (15).

In vivo activity of AMG 596 was shown in two xenograft tumor models in NOD/SCID mice. In an orthotopic intracranial tumor model, AMG 596 prolonged survival, with up to 73% of mice surviving to study end. In a second tumor model, AMG 596 treatment induced regression of established subcutaneous GBM tumors. Both studies indicated that AMG 596 can be a promising therapy for GBM.

Unlike the implanted tumors in the xenograft study, GBM tumors in humans have been reported to show heterogeneous EGFRvIII expression (26, 27). *In vitro* observations from this study showed that AMG 596 only redirected T cells in the presence of EGFRvIII-expressing cells, suggesting that only glioblastoma cells carrying the EGFRvIII mutation are suitable targets for AMG 596–mediated cell lysis, leaving the surrounding EGFRvIII-negative tumor cells unaffected. This view is supported by publications demonstrating that the BiTE molecule–triggered formation of a cytotoxic synapse between tumor and T cells and the subsequent release of cytotoxic granules exclusively induces the death of tumor cells involved in synapse formation (28, 29). However, another EGFRvIII-targeted CD3-bispecific molecule demonstrated potent killing of patient-derived explants *ex vivo*, even with heterogeneity of EGFRvIII expression (30). We propose an additional potential mechanism of AMG 596–induced redirected lysis of tumor cells that do not endogenously express EGFRvIII. We showed that EGFRvIII-negative tumor cells acquired EGFRvIII on their cell surface after the uptake of EGFRvIII-positive microvesicles isolated from EGFRvIII-expressing tumor cells. AMG 596 was not only able to bind to vesicle-transferred EGFRvIII, but also mediated lysis of EGFRvIII-transferred tumor cells. Comparable results have not been observed with EGFRvIII-negative GBM cells previously cocultured with EGFRvIII-positive GBM cells separated by a cell impermeable membrane (Supplementary Fig. S6), indicating that the microvesicle transfer requires close proximity of the two cell types. Future studies are needed to clarify whether the above-described mechanism is specific to the EGFRvIII target and is exclusively implicated in GBM or whether it is a general feature of solid tumors.

Altogether, our studies demonstrate that AMG 596 is a very potent and specific BiTE molecule with impressive *in vivo* anti-tumor activity and high tolerability in cynomolgus monkeys. These properties make AMG 596 a very promising immunotherapy for patients with GBM, a devastating and, thus far, incurable disease. A phase I study with AMG 596 is open (NCT03296696) and includes combination with an anti-programmed cell death (PD)-1 antibody to circumvent immunosuppression potentially caused by the up-regulation of PD-1 on BiTE molecule–activated T cells (31).

Authors' Disclosures

A. Sternjak reports employment with and stock ownership in Amgen. F. Lee reports employment with and stock ownership in Amgen. O. Thomas reports employment with and stock ownership in Amgen. M. Balazs reports stock ownership in and prior employment with Amgen. J. Wahl reports employment with and stock ownership in Amgen. G. Lorenczewski reports employment with and stock ownership in Amgen. I. Ullrich reports a patent for US20170029512A1 issued and employment with and stock ownership in Amgen. M. Muenz reports a patent for antibody constructs for EGFRVIII and CD3 antibody constructs for EGFRVIII and CD3 issued and employment with and stock ownership in Amgen. B. Rattel reports employment with and stock holder of Amgen. J.M. Ballis reports employment with and holds stock in Amgen Inc. M. Friedrich reports employment with and stock ownership in Amgen.

Authors' Contributions

A. Sternjak: Conceptualization, investigation, methodology, writing–original draft. **F. Lee:** Investigation, methodology, writing–review and editing. **O. Thomas:** Investigation, methodology, writing–review and editing. **M. Balazs:** Investigation, methodology, writing–review and editing. **J. Wahl:** Investigation, methodology, writing–review and editing. **G. Lorenczewski:** Investigation, methodology, writing–review and editing. **I. Ullrich:** Investigation, methodology, writing–review and editing. **M. Muenz:** Investigation, methodology, writing–review and editing. **B. Rattel:** Investigation, methodology, writing–review and editing. **J.M. Ballis:** Investigation, methodology, writing–review and editing. **M. Friedrich:** Investigation, methodology, writing–review and editing.

Acknowledgments

We thank Sid Roychoudhury, Sabine Stienen, and Angela Coxon for critical review of the article. Assistance with writing and editing the article was provided by Allison R. Gillies, PhD (ICON), Lee B. Hohaia, PharmD (ICON), and Christopher Nosala, PhD (Amgen, Inc.) and was funded by Amgen Inc. This study was funded by Amgen Inc.

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

Received June 22, 2020; revised November 25, 2020; accepted February 11, 2021; published first February 25, 2021.

References

1. Ostrom QT, Gittleman H, Xu J, Kromer C, Wolinsky Y, Kruchko C, et al. CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2009–2013. Neuro Oncol 2016;18:v1–v75.
2. Urbanska K, Sokolowska J, Szmidt M, Sysa P. Glioblastoma multiforme - an overview. Contemp Oncol 2014;18:307–12.
3. Philips A, Henshaw DL, Lamburn G, O'Carroll MJ. Brain tumours: rise in glioblastoma multiforme incidence in England 1995–2015 suggests an adverse environmental or lifestyle factor. J Environ Public Health 2018;2018:7910754.
4. Alexander BM, Cloughesy TF. Adult glioblastoma. J Clin Oncol 2017;35:2402–9.
5. Bi WL, Beroukhim R. Beating the odds: extreme long-term survival with glioblastoma. Neuro Oncol 2014;16:1159–60.
6. Yang J, Yan J, Liu B. Targeting EGFRvIII for glioblastoma multiforme. Cancer Lett 2017;403:224–30.
7. Aldape KD, Ballman K, Furth A, Buckner JC, Giannini C, Burger PC, et al. Immunohistochemical detection of EGFRvIII in high malignancy grade astrocytomas and evaluation of prognostic significance. J Neuropathol Exp Neurol 2004;63:700–7.
8. Rosenthal M, Curry R, Reardon DA, Rasmussen E, Upreti VV, Damore MA, et al. Safety, tolerability, and pharmacokinetics of anti-EGFRvIII antibody-drug conjugate AMG 595 in patients with recurrent malignant glioma expressing EGFRvIII. Cancer Chemother Pharmacol 2019;84:327–36.
9. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-

directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. Sci Transl Med 2017;9:eaaa0984.
10. Haas C, Krinner E, Brischwein K, Hoffmann P, Lutterbüse R, Schlereth B, et al. Mode of cytotoxic action of T cell-engaging BiTE antibody MT110. Immunobiology 2009;214:441–53.
11. Dufner V, Sayehli CM, Chatterjee M, Hummel HD, Gelbrich G, Bargou RC, et al. Long-term outcome of patients with relapsed/refractory B-cell non-Hodgkin lymphoma treated with blinatumomab. Blood Adv 2019;3:2491–8.
12. Bargou R, Leo E, Zugmaier G, Klinger M, Goebeler M, Knop S, et al. Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. Science 2008;321:974–7.
13. Hummel H-D, Kufer P, Grüllich C, Deschler-Baier B, Chatterjee M, Goebeler M-E, et al. Phase 1 study of pasotuxizumab (BAY 2010112), a PSMA-targeting bispecific T cell engager (BiTE) immunotherapy for metastatic castration-resistant prostate cancer (mCRPC). J Clin Oncol 2019;37:5034.
14. Friedrich M, Raum T, Lutterbuese R, Voelkel M, Deegen P, Rau D, et al. Regression of human prostate cancer xenografts in mice by AMG 212/BAY2010112, a novel PSMA/CD3-bispecific BiTE antibody cross-reactive with non-human primate antigens. Mol Cancer Ther 2012;11:2664–73.
15. Lutterbuese R, Raum T, Kischel R, Hoffmann P, Mangold S, Rattel B, et al. T cell-engaging BiTE antibodies specific for EGFR potently eliminate KRAS- and BRAF-mutated colorectal cancer cells. Proc Natl Acad Sci U S A 2010;107:12605–10.
16. Hamblett KJ, Kozlosky CJ, Siu S, Chang WS, Liu H, Foltz IN, et al. AMG 595, an anti-EGFRvIII antibody-drug conjugate, induces potent antitumor activity against EGFRvIII-expressing glioblastoma. Mol Cancer Ther 2015;14:1614–24.
17. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the care and use of laboratory animals. 8th ed. Washington, D.C.: National Academies Press; 2011.
18. Friedrich M, Henn A, Raum T, Bajtus M, Matthes K, Hendrich L, et al. Preclinical characterization of AMG 330, a CD3/CD33-bispecific T-cell-engaging antibody with potential for treatment of acute myelogenous leukemia. Mol Cancer Ther 2014;13:1549–57.
19. Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol 2008;10:619–24.
20. Einsele H, Borghaei H, Orlowski RZ, Subklewe M, Roboz GJ, Zugmaier G, et al. The BiTE (bispecific T-cell engager) platform: development and future potential of a targeted immuno-oncology therapy across tumor types. Cancer 2020;126:3192–201.
21. Hipp S, Tai Y-T, Blanset D, Deegen P, Wahl J, Thomas O, et al. A novel BCMA/CD3 bispecific T-cell engager for the treatment of multiple myeloma induces selective lysis *in vitro* and *in vivo*. Leukemia 2017;31:2278.
22. Gan HK, Cvrljevic AN, Johns TG. The epidermal growth factor receptor variant III (EGFRvIII): where wild things are altered. FEBS J 2013;280:5350–70.
23. Garcia de Palazzo IE, Adams GP, Sundareshan P, Wong AJ, Testa JR, Bigner DD, et al. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. Cancer Res 1993;53:3217–20.
24. Olapade-Olaopa EO, Moscatello DK, MacKay EH, Horsburgh T, Sandhu DPS, Terry TR, et al. Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. Br J Cancer 2000;82:186–94.
25. Pedersen MW, Meltorn M, Damstrup L, Poulsen HS. The type III epidermal growth factor receptor mutation. Biological significance and potential target for anti-cancer therapy. Ann Oncol 2001;12:745–60.
26. Del Vecchio CA, Giacomini CP, Vogel H, Jensen KC, Florio T, Merlo A, et al. EGFRvIII gene rearrangement is an early event in glioblastoma tumorigenesis and expression defines a hierarchy modulated by epigenetic mechanisms. Oncogene 2013;32:2670–81.
27. Nishikawa R, Sugiyama T, Narita Y, Furnari F, Cavenee WK, Matsutani M. Immunohistochemical analysis of the mutant epidermal growth factor, deltaEGFR, in glioblastoma. Brain Tumor Pathol 2004;21:53–6.
28. Brischwein K, Parr L, Pflanz S, Volkland J, Lumsden J, Klinger M, et al. Strictly target cell-dependent activation of T cells by bispecific single-chain antibody constructs of the BiTE class. J Immunother 2007;30:798–807.
29. Offner S, Hofmeister R, Romaniuk A, Kufer P, Baeuerle PA. Induction of regular cytolytic T cell synapses by bispecific single-chain antibody constructs on MHC class I-negative tumor cells. Mol Immunol 2006;43:763–71.
30. Gedeon PC, Schaller TH, Chitneni SK, Choi BD, Kuan C-T, Suryadevara CM, et al. A rationally designed fully human EGFRvIII:CD3-targeted bispecific antibody redirects human T cells to treat patient-derived intracerebral malignant glioma. Clin Cancer Res 2018;24:3611–31.
31. Paweletz KL, Li S, Bailis JM, Juan G. Combination of AMG 160, a PSMA x CD3 half-life extended bispecific T-cell engager (HLE BiTE) immune therapy, with an anti-PD-1 antibody in prostate cancer (PCa). J Clin Oncol 2020;38:155.