Characterization of a Novel FLT3 BiTE® Antibody Construct for the Treatment of Acute Myeloid Leukemia

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Abstract

Despite advances in the treatment of acute myeloid leukemia, novel therapies are needed to induce deeper and more durable clinical response. Bispecific T cell Engager (BiTE®) molecules, which redirect patient T cells to lyse tumor cells, are a clinically-validated modality for hematologic malignancies. Due to broad AML expression and limited normal tissue expression, FLT3 is proposed to be an optimal BiTE® molecule target. Expression profiling of FLT3 was performed in primary AML patient samples and normal hematopoietic cells and non-hematopoietic tissues. Two novel FLT3 BiTE® molecules, one with a half-life extending (HLE) Fc moiety and one without, were assessed for T-cell dependent cellular cytotoxicity (TDCC) of FLT3-positive cell lines in vitro, in vivo, and ex vivo. FLT3 expression was detected on the surface of most primary AML bulk and leukemic stem cells but only a fraction of normal hematopoietic stem and progenitor cells. FLT3 protein detected in non-hematopoietic cells was cytoplasmic. FLT3 BiTE® molecules induced TDCC of FLT3-positive cells in vitro, reduced tumor growth and increased survival in AML mouse models in vivo. Both molecules exhibited reproducible pharmacokinetic and pharmacodynamic profiles in cynomolgus monkeys in vivo, including elimination of FLT3-positive cells in blood and bone marrow. In ex vivo cultures of primary AML samples, patient T cells induced TDCC of FLT3-positive target cells. Combination with PD-1 blockade increased BiTE® activity. These data support the clinical development of a FLT3 targeting BiTE® molecule for the treatment of AML.
Introduction

Newly approved targeted therapies and cytotoxic agents (1) provide opportunities to improve treatment of acute myeloid leukemia (AML), a disease characterized by low survival rates (2). However, these therapeutics are approved for only certain patient subsets, and treatments to benefit broad patient populations are still needed. To date, the most efficacious treatment consists of intensive chemotherapy followed by allogeneic hematopoietic stem cell transplantation (HSCT) (3,4). The potent antileukemic effect of HSCT is driven by recognition and elimination of allogeneic antigens on chemoresistant leukemic cells by donor T cells. HSCT, as well as donor lymphocyte infusions, which frequently result in durable complete remissions (3), demonstrate the potential for therapies driven by T cell cytotoxicity (4). However, this regimen may not be an option for all patients due to comorbidities and the high morbidity and mortality rates associated with graft-versus-host disease, highlighting the urgent need for novel therapies (4).

A promising T cell-based therapeutic approach is to redirect a patient’s own T cells to eliminate leukemic cells. This strategy can be accomplished with bispecific T cell engaging (BiTE®) antibody constructs. BiTE® molecules consist of a single chain variable fragment (scFv) against a cell surface-expressed tumor associated antigen (TAA) linked to an scFv against the T cell co-receptor CD3. Clinical proof of concept for this modality was demonstrated by the CD19-directed BiTE® molecule blinatumomab, which is approved for B-cell precursor acute lymphoblastic leukemia. CD19 is an ideal target for a BiTE® antibody construct because it is broadly expressed on B cell malignancies, its off-tumor expression is limited to normal B cells, and patients can tolerate prolonged B cell depletion. The successful translation of BiTE® antibody constructs to AML therapy requires identification of a suitable cell surface antigen, one
that is broadly and selectively expressed by leukemic cells with limited expression on normal tissues.

FLT3 (fms related tyrosine kinase 3, CD135) is a lineage-associated growth factor that was previously reported to be expressed on AML blasts and LSCs (5,6). Expression of FLT3 on normal hematopoietic cells has been reported to be restricted to a subset of hematopoietic stem and progenitor cells (HSPCs) in the bone marrow (BM) (7). These data suggest a favorable expression profile for targeting FLT3 with a BiTE® antibody construct. Mutations in the intracellular portion of FLT3, resulting in constitutive activation, occurring as either internal tandem duplication or point mutations in the tyrosine kinase domain have been identified in approximately 25% or 7-10% of AML patients, respectively. (8-10). Tyrosine kinase inhibitors (TKIs) that target the FLT3 kinase domain were recently approved for patients with mutant FLT3 and others are undergoing clinical evaluation (11-13). FLT3 TKIs are active primarily in the setting of mutant FLT3, while BiTE® molecules recognize an extracellular protein epitope and bind FLT3 regardless of mutational status.

Here, FLT3 was evaluated as a target for BiTE® molecule therapy for the treatment of AML, including expression analysis on disease and normal cells, and two novel FLT3 BiTE® molecules were characterized in vitro, ex vivo, and in vivo. Cell surface FLT3 protein expression was observed on most primary AML (pAML) patient bulk and LSC samples, irrespective of FLT3 mutational status. Importantly, comparable FLT3 protein expression was observed on patient samples collected at the time of both initial diagnosis and relapse, suggesting a FLT3 BiTE® molecule could provide benefit to patients across multiple lines of therapy. FLT3 transcript and protein expression was rigorously evaluated in a panel of normal human tissues, and cell surface FLT3 protein was detected only on a portion of hematopoietic stem and progenitor cells and on
rare, scattered cells in the tonsil. FLT3 protein was also detected in some non-hematopoietic tissues, including cerebellum and pancreas; however, extensive characterization revealed that the protein was cytoplasmic. Because FLT3 BiTE® molecules selectively bind to cells expressing cell surface FLT3, cells expressing cytoplasmic FLT3 protein would not be expected to be depleted.

Two FLT3 BiTE® molecules were generated and evaluated: An experimental FLT3 BiTE® molecule comprised an anti-CD3 scFv and an anti-FLT3 scFv, and a FLT3 HLE BiTE® molecule (AMG 427) comprised an anti-CD3 scFv fused to a half-life extending Fc moiety and a unique anti-FLT3 scFv. Due to the size of the experimental FLT3 BiTE® molecule, rapid clearance by glomerular filtration is expected to result in a short serum half-life, requiring continuous intravenous (cIV) infusion to maintain an active concentration in vivo. The larger AMG 427 was designed to have an extended serum half-life relative to the experimental FLT3 BiTE® molecule. Both BiTE® molecules induced potent and target-specific T cell-dependent cellular cytotoxicity (TDCC) against AML cell lines in vitro, inhibited tumor growth and provided a survival advantage in vivo in xenograft models and exhibited reproducible pharmacokinetic (PK) and pharmacodynamic (PD) profiles in cynomolgus monkeys. The experimental FLT3 BiTE® molecule induced TDCC of patient AML (pAML) samples ex vivo. Increased in vitro TDCC was observed by combining AMG 427 with an anti-PD-1 antibody. These data demonstrate that FLT3 BiTE® molecules are capable of inducing TDCC of FLT3-expressing cells in vitro, in vivo, and ex vivo; moreover, while each FLT3 BiTE® molecule was efficacious as a single agent against AML cell lines and pAML samples, combination therapy may provide additional benefit for some patients. AMG 427 is being evaluated patients with relapsed or refractory AML.
Materials and Methods

Patient and Healthy Donor Samples

AML and healthy donor (HD) samples were obtained with written informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig-Maximilian University. (Tables S1, S2). Human tissue specimens for expression analyses were collected under Institutional Review Board approval with appropriate informed consent. In all cases, materials obtained were surplus to standard clinical practice. Patient identity and protected health information/identifying information were redacted from tissue data and clinical data.

Key Resources

Sources of biological samples, all antibodies and other key reagents are listed in Table S1.

FLT3 Protein Expression on AML Patient and HD Hematopoietic Cells

Cell surface FLT3 protein expression on AML patient and HD peripheral blood (PB) or bone marrow (BM) samples was assessed by flow cytometry (Navios, Beckman Coulter) using an anti-FLT3 antibody (Table S1). Mean fluorescence intensity was determined (FlowJo version 10.3) and the MFI ratio (MFI sample/MFI isotype control) was calculated.

FLT3 Transcript Expression in AML Patient Cells and HD Non-Hematopoietic Tissues

FLT3 transcript expression data were retrieved from The Cancer Genome Atlas (TCGA (14), AML patient samples) in February 2018.
FLT3 transcript expression data in normal human tissues were retrieved from the Genotype-Tissue Expression project (GTEx (15), HD samples) in April 2018.

'5' rapid amplification of cDNA ends (RACE), digital droplet polymerase chain reaction (ddPCR), reverse transcription PCR, immunohistochemistry, Western analysis, immunoprecipitation, and RNA-seq were conducted using standard techniques. Details in Table S1 and Supplementary Methods.

AML Cell Lines: Cytotoxicity, T Cell Activation, Cytokine Secretion

Cell lines were initially sourced from DSMZ (MOLM-13, EOL-1, PL-21), ATCC (HL-60, MV4-11, K562, HEL92.1) and ECACC (A2780), and cultured using standard techniques and reagents. In the absence of phenotypic or growth changes, cells were not authenticated or tested for mycoplasma. Cells were used within two months of thawing.

Human PBMCs or pan T cells were cultured for 48 hours in the presence or absence of FLT3 expression–positive or -negative target cells with an effector-to-target (E:T) cell ratio of 10:1 (pan T) or 5:1 (PBMC) and a dose range of FLT3 BiTE® molecules. Target cell lysis was measured by loss of luciferase signal (Steady-Glo™, Promega; labeled target cell lines express luciferase); or propidium iodide uptake by flow cytometry. T cell activation markers were assessed by flow cytometry. Antibodies against CD4, CD8, CD69, and CD25 were labeled with a fluorochrome conjugate (Table S1). BiTE®-induced cytokine secretion was measured in supernatants using the BD™ Cytometric Bead Array Human Th1/Th2 Cytokine Kit. Luciferase-based TDCC (pan T, E:T ratio 10:1) was performed with or without 10 ng/mL soluble FLT3 ligand (16) for 48 hours.
Mouse Xenograft Models

Animal experimental procedures were conducted in accordance with the German Animal Welfare Law with permission from the responsible local authorities and within the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) international standards.

Female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice at age 7 weeks were sublethally irradiated prior to tumor cell injection. Mice were injected intravenously via lateral tail vein with \(10^7\) MOLM-13_Luc or \(5 \times 10^6\) EOL-1 cells on day 1. After 48 or 72 hours, respectively, mice were injected intraperitoneally with human \emph{in vitro}–expanded CD3\(^{+}\) T cells (2 or 1.2 \(\times 10^7\), respectively) and allocated to treatment groups (n=10/group). Five mice allocated to the vehicle group did not receive human T cells. Mice were treated with vehicle or AMG 427 (3, 0.6, or 0.12 mg/kg) every 5 days by IV bolus injection into the lateral tail vein starting on day 7, then on days 12, 17, 22, 27, and 34 (MOLM-13_Luc). Mice were treated with vehicle or AMG 427 (1, 0.1, or 0.001 mg/kg) every 7 days by IV bolus injection into the lateral tail vein starting on day 9 for a total of six administrations (EOL-1). To block binding of AMG 427 to Fc receptors, a mixture of anti-muFcRII (2.4G2) antibody (8 mg/kg) and human normal immunoglobulin (400 mg/kg of Kiovig\textsuperscript{®}) was administered once weekly intraperitoneally throughout the treatment period, starting 1 day prior to the first AMG 427 dose. Mice were monitored daily. PK serum concentrations of AMG 427 were determined by electrochemiluminescence immunoassay (Supplementary Methods).

Cynomolgus Monkey Studies
Cynomolgus monkeys were cared for in accordance to the Guide for the Care and Use of Laboratory Animals, Eighth Edition (17). Animal care is detailed in Supplemental Methods.

Cell surface FLT3 protein expression on hematopoietic cells from cynomolgus monkeys was assessed as described in the Supplementary Methods.

The experimental FLT3 BiTE® molecule was evaluated in a 16-day cIV study (n=3) and was administered at step doses increasing every 3 days, intended to achieve $C_{ss}$ of 0.05, 0.2, 0.5, and 2 nM for 3 days each. Methods for PK and PD assessment are detailed in Supplementary Methods. AMG 427 was evaluated in an 8-day, repeated-dose study with three dose levels (n=3/group). AMG 427 was administered intravenously on days 1, 2, and 5.

**Cytotoxicity Against pAML Cells**

AML patient samples were cultured (Supplementary Methods) with experimental FLT3 BiTE® molecule or control BiTE® molecule at 5 ng/mL (92 pM) and replenished at 3-day intervals. Viable CD33⁺/CD2⁻ cells (Table S1) were determined by flow cytometry, and total cell count was used to determine AML cell count.

**Combination With PD-1–Blocking Antibody**

Human pan T cells were stimulated 1:1 with CD3/CD28 Dynabeads (Thermo Fisher) for 48 hours, then co-cultured 1:1 with PD-L1–transfected MOLM-13 cells (MOLM-13_PD-L1) and dose range of AMG 427 in the absence or presence of 10 µg of a PD-1–blocking antibody (Table S1). After 24 hours, MOLM-13_PD-L1 cell viability was determined by TO-PRO-3 uptake by flow cytometry.
Novel FLT3 BiTE® Molecule for AML treatment
Results

AML Patient Sample Cell Surface FLT3 Protein Expression

Leukemic bulk cells from BM or peripheral blood (PB) from 318 newly diagnosed or relapsed AML patients were evaluated for cell surface FLT3 protein expression. Of the analyzed samples, 78% (248/318) were positive for FLT3 protein expression (mean fluorescence intensity [MFI] ratio > 1.5; Fig. 1A, gating strategy Fig. S1A upper panel, MFI ratio calculation Fig. S1B). Inter-patient heterogeneity in FLT3 protein expression was observed (MFI ratio range 0.1-32.7; Fig. 1A), similar to what has been reported for other AML-associated antigens (18). The FLT3 protein expression profile was similar, regardless of FLT3-ITD mutational status (Fig. 1B), time of sample collection (initial diagnosis versus relapse, Fig. 1C), or FLT3-ITD allelic ratio (Fig. 1D). Cell surface FLT3 protein expression was detected on leukemic stem cells (LSC; CD34+/CD38-) in 79% (122/155) of AML patient samples (Fig. 1E). As observed for bulk cells, FLT3 protein expression on LSCs was similar, regardless of FLT3-ITD mutational status (Fig. 1F) or initial diagnosis versus relapse (Fig. 1G). Higher FLT3 expression was detected on samples with high FLT3-ITD allelic ratio (Fig. 1H, p<0.0098).

No clear correlation was observed in an analysis of FLT3 protein expression intensity on AML patient bulk cells at initial diagnosis with different disease characteristics, including French American British (FAB) group, core binding factor (CBF) abnormalities (i.e., translocation t(8;21) and inversion inv(16)), nucleophosmin 1 (NPM1) and FLT3-ITD mutations, Medical Research Council (MRC) cytogenetic based risk classification (19), and 2010 European Leukemia Net (ELN) classification (20) (Fig. S1C-F, Table S2).
FLT3 Transcript and Protein Expression in Normal Human Hematopoietic Cells

In hematopoietic cell samples derived from healthy donors (HD), the FLT3 protein MFI ratio was consistently low and less than that of pAML samples. The MFI ratio on HD CD34⁺CD38⁻ cells (n=18), comprising hematopoietic stem cells and multipotent progenitors, was 0.58 ± 0.26 and the MFI ratio on CD34⁺CD38⁺ cells (n=36), comprising the oligopotent progenitors, was 1.6 ± 0.5 (Fig. 1I; gating strategy Fig. S1A lower panel, MFI ratio calculation Fig. S1B). In comparison, the MFI ratio for pAML samples was significantly higher than either of the HD samples (p < 0.0001 comparison to either HD CD34⁺CD38⁻ cells or HD CD34⁺CD38⁺ cells) at 3.6 ± 3.6 for the bulk samples (n=318) and 2.9 ± 2.3 for the LSC samples (n=155). Paired analysis of CD34⁺CD38⁻ and CD34⁺CD38⁺ cells from 13 healthy donors showed that FLT3 protein expression was statistically lower on CD34⁺CD38⁻ than CD34⁺CD38⁺ cells (Fig. 1I).

FLT3 protein expression was subsequently evaluated on individual stem cell and oligopotent progenitor subsets from two HD. Expression was variable, and no subset was uniformly positive or negative (Fig. 1K, gating strategy Fig. S2A). The rank order of FLT3 protein expression was: granulocyte/macrophage progenitor (GMP) > common lymphoid progenitor (CLP) > common myeloid progenitor (CMP) > hematopoietic stem cell (HSC) and multipotent progenitor (MPP) > megakaryocyte/erythrocyte progenitor (MEP), most of the latter population falling below detection.

On mature hematopoietic cells isolated from blood from two HD, there was no detectable cell surface FLT3 protein on T or B lymphocytes, natural killer cells, plasmacytoid or conventional dendritic cells, monocytes or neutrophils (Fig. S2B). Collectively, these data demonstrate that there are differences in expression between disease and normal cells, and within hematopoietic
stem and progenitor populations, which may translate to differences in susceptibility to FLT3 BiTE®-mediated killing.

**FLT3 Transcript and Protein Expression in Normal Human Non-Hematopoietic Tissues**

The presence of *FLT3* transcript in non-hematopoietic tissues was assessed in three different datasets including GTEx RNAseq database (15), an Amgen-constructed RNAseq database, and XpressWay® Profile Report (Asterand UK Acquisition Limited, Royston, UK). Low levels of *FLT3* transcript were detected in brain, nerve/ganglia, small intestine, kidney, lung, pancreas, spleen, spinal cord, and testis (Fig. 2A, Table S3). Within the brain, *FLT3* transcripts localized to the cerebellum (Fig. 2B). While *FLT3* transcript was not consistently detected in all tissues listed above, all tissue types identified as transcript-positive in any dataset were subsequently evaluated for FLT3 protein expression by immunohistochemistry (IHC; spinal cord being the only exception). Of these tissues, the only example of cell surface-localized FLT3 protein was on rare, scattered cells in the tonsil (Fig. 2C). In all other tissues evaluated, including brain stem, cerebrum, cerebellum, kidney, pancreas, pituitary, prostate, skeletal muscle, stomach, testis, and thyroid, FLT3 protein staining was cytoplasmic (Fig. 2D). Within the cerebrum and cerebellum, FLT3 protein staining consisted of cytoplasmic staining of multifocal neurons, and this staining pattern was consistent in multiple sections of brain, with no membranous staining observed in neurons. Diffuse cytoplasmic staining was observed in alveolar macrophages, indicating the likely source of the transcript signal in lung (Table S3). Taken together, these data suggest that although FLT3 transcript and protein are present in peripheral tissues, including the brain, FLT3 protein is cytoplasmic and therefore not anticipated to be targeted by an anti-FLT3 BiTE® molecule.
Additional analysis of FLT3 transcript and protein expression in the cerebellum revealed that the majority of FLT3 transcripts isolated from the cerebellum were shorter than those isolated from a control AML cell line. Transcript sequencing revealed these truncations were due to frequent intron insertion/retention or exon skipping. Quantification of alternatively-spliced FLT3 transcripts using digital droplet PCR (ddPCR) indicated that in this study at least 70% to 85% of cerebellum FLT3 transcripts lacked exonic regions or retained intronic sequences, suggesting that only a small portion of FLT3 transcripts in cerebellum samples analyzed would be intact (Fig. S3). Assessment of FLT3 protein from human cerebellum lysate by immunoprecipitation-western analysis identified only FLT3 protein bands that were lower in molecular weight than full-length FLT3 protein from a positive control AML cell line lysate (Fig. 2E). FLT3 protein bands from a cerebellum sample were characterized by mass spectrometry, revealing only peptides from the extracellular domain of FLT3; by contrast, bands from the control AML cell line lysate contained multiple peptides from both the intracellular and extracellular regions of FLT3 (Fig. S3D, Table S11). In sum, the transcript and peptide data suggest that most transcripts from the cerebellum encode FLT3 peptides that are not full-length and may explain why FLT3 is not detectable on the cell surface of cells in the cerebellum.

**FLT3 BiTE® Molecules Induced TDCC of FLT3-Expressing AML Cell Lines**

Two different FLT3 BiTE® molecules (Figure S4A) were evaluated. Each BiTE® molecule comprised a distinct anti-FLT3 scFv that bound FLT3 within a 51 amino acid region, associated with an anti-CD3 scFv. The compact size of BiTE® molecules (MW ~55 kDa) has been reported to be important for the generation of a productive immunological synapse (21); however, proteins this size are generally rapidly eliminated by the kidneys. To increase the serum half-life, an Fc moiety was added to produce AMG 427. To ensure that the presence of the Fc would not
impact in vitro or in vivo activity, the two BiTE® molecules were evaluated in similar assay panels. Both molecules bound human FLT3 and CD3 protein with sub- or single-digit nanomolar affinities (Table S5). A panel of cell lines exhibiting a range of FLT3 protein expression (MFI ratio: 2.6 – 23.8; Fig. S4B) similar to that observed on primary AML samples (Fig. 1A) was selected to evaluate FLT3 BiTE® molecule in vitro potency. Both molecules similarly induced TDCC against five FLT3 protein-expressing cell lines with single digit picomolar potency (Fig. 3A, Table S6). A relationship between FLT3 expression level and potency was not apparent, likely due to the high E:T ratio. TDCC was similar for both BiTE® molecules in cell lines homozygous or heterozygous for wild type (wt) or ITD mutant (mut) FLT3, and selectivity was demonstrated as cell lines lacking FLT3 protein expression were not lysed (Fig. 3A, Table S6). TDCC was accompanied by upregulation of the T cell activation markers CD69 and CD25 and secretion of T cell-derived effector cytokines interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) in the presence of FLT3 protein-expressing cells, but not in the absence of FLT3 protein-expressing cells (Fig. 3B-3E, Table S6).

Soluble FLT3 (sFLT3) can be detected in AML patient serum at concentrations up to 141 ng/mL (22). In TDCC assays, clinically relevant concentrations of sFLT3 reduced AMG 427 potency 6-44-fold, but maximum killing was still achieved (Fig. 3F). Soluble FLT3 ligand (sFLT3L) can be detected in AML patient serum at concentrations up to 9 ng/mL (23). Although neither the experimental FLT3 BiTE® molecule nor AMG 427 binds the ligand-binding domain of FLT3, sFLT3L binding to FLT3 induces internalization of FLT3 (24), and could alter BiTE®-mediated TDCC. In the presence of 10 ng/ml sFLT3L, the potency of AMG 427-mediated TDCC was reduced 2-6-fold (Fig. 3F); however, maximum killing was still achieved in all three cell lines tested. These data demonstrate that FLT3 BiTE® molecules induce target-specific TDCC.
equivalently, and that complete killing occurs in the presence of disease-relevant concentrations of sFLT3 and sFLT3L.

**Experimental FLT3 BiTE® Molecule and AMG 427 Inhibited Tumor Growth and Increased Survival in Mouse Xenograft Models**

Both the experimental FLT3 BiTE® molecule and AMG 427 were evaluated in mouse tumor models. As neither BiTE® molecule bound mouse FLT3, immunocompromised mice administered with human tumor cells and T cells were used. The experimental FLT3 BiTE® molecule was evaluated in an admix model in which athymic nude mice were injected with MOLM-13 AML cells and *in vitro*-expanded human CD3⁺ T cells in Matrigel. Animals were dosed intraperitoneally (IP) with experimental FLT3 BiTE® molecule or control BiTE® molecule daily for 10 days. Tumor growth was inhibited by 90% in mice treated with the experimental FLT3 BiTE® molecule relative to the control BiTE® molecule (n=10, p < 0.0001; Fig. S5).

AMG 427 was evaluated in two orthotopic mouse xenograft models in which either EOL-1 or MOLM-13 AML cells were injected on day 1 and after 72 or 48 hours (EOL-1 and MOLM-13, respectively), mice were injected with *in vitro*-expanded human CD3⁺ T cells. Mice were treated with vehicle or AMG 427 every 7 days starting on day 9 (EOL-1) or every 5 days starting on day 7 (MOLM-13). In the EOL-1 model, all animals from the control groups developed leukemic disease and were euthanized between days 27 and 52 following AML cell injection with median survival of 36 and 37 days (Fig. 4A). Weekly treatment with AMG 427 prolonged survival at all doses tested, with 17/30 animals surviving until study end on day 108. As ≥ 50% of animals were alive at study end, the median survival could not be calculated; however, compared to vehicle, AMG 427 significantly extended survival (n=10, p < 0.001; Fig. 4A).
aggressive MOLM-13 model, all mice in the control groups died within 20 days after injection of AML cells, with median survival of 18 days. Compared to vehicle, treatment with AMG 427 significantly extended survival at all doses tested (n=10, p ≤ 0.0015, Fig. 4B). No significant difference in overall survival was observed between different dose levels. Comparable PK profiles were observed within each cohort for all dose levels, and serum concentrations remained above the TDCC assay-determined EC₅₀ for at least 9 days following the final administration (days 34-43, Fig. 4C). Serum half-life of AMG 427 ranged from 33 to 47 hours. These data demonstrate that both FLT3 BiTE® molecules were active in vivo in mouse tumor models.

**PK and PD Profile of FLT3 BiTE® Molecules in Cynomolgus Monkeys**

The experimental FLT3 BiTE® molecule and AMG 427 bound human and cynomolgus monkey FLT3 and CD3 protein with comparable affinity (Table S5). BiTE®-induced TDCC was similar for both constructs using either cynomolgus monkey or human effector cells (Fig. 3, Fig. S6). To assess the PK/PD relationship, both FLT3 BiTE® molecules were evaluated in vivo in cynomolgus monkeys. Both molecules were well tolerated. PD endpoints included FLT3 transcript levels (primer and probes in Table S9) in BM and blood and circulating sFLT3L levels. Reduction of FLT3 transcript levels in BM was likely due to direct killing of FLT3 transcript-expressing hematopoietic progenitor cells, and reduction in the blood was likely due to lack of replenishment of FLT3 transcript-expressing cells from the BM. This hypothesis is supported by data showing that there are cells in the BM that express both FLT3 transcript (Fig. 5B, 5F) and surface-localized FLT3 protein (Fig. S7B), making them recognizable by FLT3 BiTE® molecules, whereas none of the FLT3 transcript-expressing cells in blood express detectable surface-localized FLT3 protein (Fig. S7A), and are therefore not recognizable by FLT3 BiTE® molecules.
The experimental FLT3 BiTE® molecule was evaluated in a 16-day study (Fig. 5A-D) in cynomolgus monkeys, with intra-animal (n=3) dose escalations every 3 days intended to achieve steady-state concentrations ($C_{ss}$) of 0.05, 0.2, 0.5 and 2 nM (Fig. 5A, Table S8). FLT3 transcript levels were reduced in bone marrow at day 17 (the only time point evaluated) relative to non-treated animals (Fig. 5B) and in blood on days 4, 7, 10, and 17, by an average of 85% to 92%, relative to levels measured before treatment (Fig. 5C). Soluble FLT3L levels increased dose-dependently over the course of the study, reaching maximum levels of 13,000-15,500 pg/ml at the end of the study (Fig. 5D). Ligand accumulation is likely due to depletion of FLT3 protein-expressing cells. The fold-over-EC$_{50}$ (in vitro TDCC data, Fig. S6) for each of the four dose levels ($C_{ss}$~0.05, 0.2, 0.5 and 2 nM) was 25-, 64-, 165- and 780-fold. The percent reduction in FLT3 transcript level in blood did not deepen once drug concentration was above $C_{ss}$ 0.2 nM (64-fold-over-EC$_{50}$), suggesting that the concentration required to achieve maximal target cell elimination from blood was somewhere between $C_{ss}$ 0.05 and 0.2 nM (25- and 64-fold-over-EC$_{50}$, TDCC data Fig. S6, Table S7).

AMG 427 was evaluated in an 8-day multiple dose study (Fig. 5E-H) in cynomolgus monkeys. All animals were treated on days 1, 2, and 5 with doses intended to achieve a maximal serum concentration ($C_{max}$) of 1 nM (Group 1), 5 nM (Group 2), and 10 nM (Group 3; n=3/group). The study duration was limited to 8 days to minimize loss of exposure due to anti-drug antibody formation, and multiple doses were administered to ensure target coverage for the entire study. Exposures of AMG 427 over 7 days were reproducible within each of three dose groups (Fig. 5E), and exposure, $C_{max}$, and $C_{min}$ all increased in an approximately dose-proportional manner (Table S8). The terminal half-life ranged from 33-50 hours (Table S9). Hallmarks of BiTE® molecule activity including upregulation of CD69 on T cells and cytokine secretion were
observed (Fig. 8A-B). Because FLT3 is not expressed on the surface of cells in the blood (Fig. S7), AMG 427-mediated upregulation of CD69 on T cells likely resulted from cells expressing FLT3 surface protein in the bone marrow. Increases in serum concentrations of IFNγ, IL-6, MCP-1, and TNFα were observed in response to the first dose but were attenuated in response to subsequent doses (Fig S8B). The fold-over-EC\textsubscript{50} levels at C\textsubscript{min} \textit{(in vitro} TDCC data Fig. S6, Table S9) for the respective groups was 45-, 158-, and 396-fold. Within the bone marrow, \textit{FLT3} transcript levels were reduced by 85-95% on day 4 and by 93-97% on day 8 (Fig. 5F). Within the blood, the \textit{FLT3} transcript levels were reduced to a nearly undetectable level (≥ 97%) at the lowest dose level and earliest time point, and a similar level of depletion was maintained across all higher exposures and time points (Fig. 5G). Monocytes were reduced at the end of the study (Fig. S8C), which may reflect lack of replenishment due to direct killing of bone marrow progenitors. Minor decreases in plasmacytoid dendritic cells (pDCs, Fig. S8C) are challenging to interpret as the number of circulating dendritic cells was low and enumeration of rare cells is prone to error. Soluble FLT3L levels increased dose-dependently over the course of the study, reaching maximum levels of 12,000-23,000 pg/ml in each of the three groups (Fig. 5H). Time-dependent improvements in PD were observed for those endpoints which hadn’t already reached maximal levels when first analyzed, as demonstrated by the increase in \textit{FLT3} transcript reduction in BM from group 1 on day 4 (85%) to day 8 (93%, Figure 5F) and changes in sFLT3L (Fig. 5H, Table S9). This demonstrates that greater efficacy at a given dose level may be observed with longer duration of exposure.

The \textit{in vivo} activity of the experimental FLT3 BiTE\textsuperscript{®} molecule and AMG 427 was most directly comparable using the PD endpoint of \textit{FLT3} transcript in blood. For the experimental FLT3 BiTE\textsuperscript{®} molecule, the greatest activity occurred between days 4 and 7 at a C\textsubscript{ss} of 0.05-0.2 (25-
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64-fold-over-EC$_{50}$, Table S8). For AMG 427, the greatest activity was observed on day 3 at a $C_{\text{min}}$ of 0.24 nM in Group 1 ($\leq 45$-fold-over-EC$_{50}$, Table S9). Although the time points of data collection differed, these results suggest that both FLT3 BiTE® molecules are active at similar fold-over-EC$_{50}$ values in vivo.

Experimental FLT3 BiTE® Molecule-Induced TDCC of Patient Samples Ex Vivo

A long-term culture system (25) was used to evaluate experimental FLT3 BiTE® molecule-mediated cytotoxicity in 14 pAML samples (Table S10) over 9 days. The autologous E:T ratio was calculated from the number of T cells and pAML cells in each sample at the beginning of the experiment and ranged from 1:2.5 to 1:74. Three patterns of cytotoxicity were observed: (1) continuously-increasing cytotoxicity (Fig. 6A left, representative sample Fig. S9A); (2) initial cytotoxicity followed by sustained or decreased killing (Fig. 6A middle); (3) transient or no cytotoxicity over the 9 days (Fig. 6A right). Analysis of FLT3 surface protein expression of the pAML cells and E:T ratio revealed that most of the samples in groups 1 and 2 contained FLT3 protein-positive pAML cells (MFI Ratio $> 1.5$) and a higher E:T ratio ($> 1:38$, 75$^{\text{th}}$ percentile; Fig. 6B), while most of the samples in group 3 expressed low levels of FLT3 protein (MFI ratio $< 2$) and/or had a low E:T ratio ($< 1:38$). These data demonstrate that both target expression and T cell abundance are important factors for FLT3 BiTE®-mediated target cell killing.

AMG 427 Potency Was Increased in Combination With a PD-1–Blocking Antibody

T cell activation induces PD-1 expression, and reports show that PD-1 engagement by ligands PD-L1 or PD-L2 decreases T cell activity (26). Co-culture of primary AML specimens with a CD33-targeting BiTE® molecule induces PD-1 expression on T cells and PD-L1 expression on AML blasts (27). Similarly, AMG 427-mediated T cell activation induced a dose-dependent
increase in PD-1 expression (Fig. S9B), and potency in TDCC assays was reduced 5-fold if the target cells expressed PD-L1 (relative to target cells lacking PD-L1, Fig. 6C). Combination of a PD-1–blocking antibody with AMG 427 restored TDCC potency, decreasing the EC$_{50}$ by an average of 2.5-fold (n=3 T cell donors, p=0.02) and increasing maximum killing by 12% (Fig. 6D, 6E). These data demonstrate that AMG 427-mediated target cell killing may be enhanced by combination with PD-1 blockade, as has been demonstrated for other BiTE® molecules (27,28).

**Discussion**

Blinatumomab demonstrates that a BiTE® molecule can engage patient T cells to eliminate CD19-expressing disease cells, and this activity can provide clinical benefit for patients with acute lymphoblastic leukemia and non-Hodgkin lymphoma (29-31). Here FLT3 BiTE® molecules for the treatment of patients with AML are characterized. FLT3 meets the requirements of a BiTE® molecule target as cell surface protein is broadly expressed on disease samples, with limited expression on normal tissues. In disease samples, cell surface FLT3 protein was detected on the majority of 318 pAML samples. The level of expression was comparable between bulk samples and LSCs suggesting that both subsets could be targeted at similar therapeutic exposures. In addition, mean FLT3 protein expression on pAML samples was comparable, regardless of FLT3 mutational status, FLT3-ITD allelic ratio, or initial diagnosis versus relapse, suggesting that a FLT3 BiTE® molecule would benefit a broad patient population. In normal hematopoietic cells, cell surface FLT3 protein was detected on subsets of bone marrow stem and progenitor cells (excluding MEPs). Within each subpopulation, a portion of cells were cell surface FLT3-positive (MFI ratio ≥ 1.5) so as to suggest their elimination by a FLT3-targeting BiTE® molecule. However, a portion of these cells were FLT3 protein-negative, consistent with literature reports of FLT3 protein– and transcript–negative cells within healthy
donor hematopoietic progenitor populations and HSCs (7,32), GEO accession code GSE75478). These results suggest that while some hematopoietic stem and progenitor cells would be eliminated by a FLT3 BiTE® molecule, there is a FLT3-negative population that could potentially repopulate the bone marrow following cessation of treatment. FLT3 transcript and protein expression were also evaluated in normal non-hematopoietic tissues. To ensure a thorough assessment, FLT3 transcript expression was evaluated in several databases and further characterized by qPCR-based analysis of a panel of tissues. Protein expression was subsequently evaluated in tissues shown to contain FLT3 transcript in any dataset. Although FLT3 transcript and protein were detected in some solid tissues, no membranous protein staining was observed, indicating that these cells would not be targeted by a FLT3 BiTE® molecule. Additional analysis of FLT3 transcript and protein in the cerebellum demonstrated that most transcripts were not full-length due to alternative splicing, and similarly, the FLT3 protein was also not full-length. By evaluating both FLT3 transcript and protein expression using multiple sources and orthogonal methods, it was possible to build a detailed understanding of the normal tissue expression and based on these results, FLT3 BiTE® treatment is not anticipated to target normal non-hematopoietic tissues.

Given the favorable expression profile of FLT3 as an AML target, two potent and specific BiTE® molecules were generated: one experimental FLT3 BiTE® molecule that has a short serum half-life and the other, AMG 427, which contains an Fc-moiety to extend serum half-life. In vitro, these molecules demonstrated TDCC against human FLT3-positive cancer cell lines with similar picomolar potency (EC\textsubscript{50}) and this TDCC was associated with T cell activation and cytokine secretion and was not affected by the presence of sFLT3 or sFLT3L at concentrations found in AML patients. These data demonstrate it is possible to generate a BiTE® molecule capable of
eliminating FLT3-expressing cells, and that despite incorporation of the Fc moiety, the larger size does not impact the ability of AMG 427 to effectively form an immunological synapse and induce T-cell–mediated target cell killing.

In cynomolgus monkeys, both FLT3 BiTE® molecules mediated depletion of cell surface FLT3-expressing target cells as demonstrated by decreases in FLT3 transcript in the blood and bone marrow. Although cell surface FLT3 protein expression was not detected on human or cynomolgus monkey peripheral immune cells, FLT3 transcript can be detected in plasmacytoid dendritic cells and monocytes. These cell types have short half-lives in vivo (33,34), and administration of a FLT3 BiTE® molecule is expected to eliminate a portion of the precursor cells that give rise to them, which may explain the decreases in FLT3 transcript observed in the blood of cynomolgus monkeys treated with a FLT3 BiTE® molecule. Within the BM, FLT3 transcript was reduced by ≥ 85% at all doses by the first timepoint tested (day 4) demonstrating that BiTE®-mediated target cell killing can occur rapidly. At this same time point, the degree of depletion increased as the dose increased (85% [low dose] vs 95% [medium dose], and 93% [high dose], respectively), suggesting that increased exposure can lead to deeper responses. Within the low dose group, the reduction in FLT3 transcript levels increased from 85% to 93% between days 4 and 8, suggesting that deeper responses may also be achieved by maintaining the same exposure for longer. This hypothesis is supported by the sFLT3L endpoint which improved with either higher exposure or increased time of exposure.

BiTE® molecule-mediated lysis of AML blasts within patient samples was evaluated in long-term culture assays using autologous T cells. The degree of anti-AML activity was associated with FLT3 expression on the target cells and the E:T ratio, with improved activity seen in AML samples with a higher FLT3 protein expression and an E:T ratio > 1:38. The impact of the E:T
ratio highlights the importance of T-cell fitness to enable successful responsiveness to BiTE® molecule therapy. One well-established mechanism of reducing T-cell activity is induction of PD-1 expression. BiTE® molecule–mediated T-cell activation is accompanied by expression of PD-1 on corresponding T cells and this expression has been associated with resistance to blinatumomab treatment (28,35). PD-1 is expressed on 20-30% of AML patient T cells (36,37) and has been shown to increase to 50-60% at relapse (38). PD-L1 mRNA expression is upregulated in AML patients (39) and correlates with cell surface protein expression (40). Although not usually detected at diagnosis (41), PD-L1 protein is upregulated on AML blasts during therapy, after HSCT, and at relapse (39,42). Upregulation of PD-L1 on AML blasts is reported to be induced by cytokines such as IFNγ (43,44), which may be the mechanism of PD-L1 upregulation on primary AML blasts treated ex vivo with a CD33-targeting BiTE® molecule (27). In a mouse model engineered to express human CD3, combination studies of BiTE® molecules with checkpoint inhibitors exhibit additive effect (45). Herein, AMG 427–mediated activation of T cells was associated with rapid induction of PD-1 expression and subsequent reduced killing of PD-L1–expressing target cells, suggesting that combination with PD-1 blockade may improve BiTE®-mediated activity. Indeed, the combination of BiTE® molecule and a PD-1–blocking antibody in a TDCC assay resulted in decreased EC50 and increased maximum killing in all donors tested. As expression of checkpoint molecules, including PD-1, has been observed in AML patients (39), and may be increased following chemotherapy (43), this combination therapy warrants clinical evaluation.

Authorship Contributions
C.K., M.B., T.R., K.H.M., K.S., and M.S. were involved in data collection. B.B., R.L.G., M.S. and T.A. drafted the manuscript with input from all authors. All authors contributed to the analysis and interpretation of data, and read, revised, and approved the final manuscript.

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**Figure Legends**

**Figure 1.** Cell surface FLT3 protein expression on AML patient bulk cells (A-D), AML LSCs (E-H), and healthy donor cells (I-K). Red line indicates FLT3 positivity (MFI ratio ≥ 1.5). (A) FLT3 protein expression on primary AML bulk cells (CD45^{DIM}/SSC^{LOW}) at initial diagnosis or relapse (n=318). Comparison of FLT3 protein expression on (B) AML bulk cells expressing wild-type *FLT3* (n=233) vs. mutant *FLT3* (ITD mutation; n=68; p=0.22), (C) AML bulk cells expressing high (≥ 0.5) *FLT3-ITD* allelic ratio (n=20) vs. low (< 0.5) *FLT3-ITD* allelic ratio (n=14; p=0.655), or samples collected at (D) initial diagnosis (n=275) vs. relapse (n=43; p=0.99). (E) FLT3 protein expression on primary AML CD34^{+}/CD38^{-} LSCs at initial diagnosis or relapse (n=155). Comparison of FLT3 expression on AML LSCs of patients with (F) wild-type *FLT3* (n=114) vs. mutant *FLT3* (ITD mutation; n=33; p=0.43), (G) LSCs expressing high (≥ 0.5) *FLT3* allelic ratio (n=13) vs. low (< 0.5) *FLT3* allelic ratio (n=5; p=0.0098), or samples collected at the time of (H) initial diagnosis (n=132) vs. relapse (n=23; p=0.37). (I) Surface FLT3 expression on HD CD34^{+}CD38^{+} (n=36) vs. patient leukemic bulk cells (n=318; p < 0.0001) and HD CD34^{+}CD38^{-} (n=18) vs. patient LSCs (n=155; p < 0.0001). (A-I) Mann Whitney U test (mean ± SEM); ns, not significant. (J) Paired analysis of FLT3 expression on HD samples (n=13; p=0.0002; Wilcoxon matched-pairs signed rank test). (K) Analysis of FLT3 protein expression on progenitor subsets. Black vertical line represents the FLT3 protein expression cutoff determined from an unstained negative control.

**Figure 2.** *FLT3* transcript expression in normal human solid tissues. *FLT3* transcript expression in (A) AML and (B) normal solid tissue shown as fragments per kilobase of transcript per million mapped reads (FPKM). Data are represented as mean ± SD for the indicated tissues. Immunohistochemistry of FLT3 protein in human (C) tonsil, (D) cerebellum, and pancreas. Low
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(left) and high (middle) magnification cerebellum and low magnification pancreas (right) images demonstrate punctate cytoplasmic immunostaining and lack of membranous staining. (E) Immunoblot of FLT3 protein immunoprecipitated from EOL-1 and human cerebellum protein lysates. Bands between 130 and 180 kDa for EOL-1 and numbered bands shown in the gel for cerebellum were isolated and analyzed by mass spectrometry (see Fig. S3D and Table S11).

**Figure 3.** FLT3 BiTE® molecules have potent cytotoxic activity against FLT3-expressing AML cell lines (A) Specific cytotoxicity of FLT3 BiTE® molecules (experimental FLT3 BiTE® molecule, left panel; AMG 427 , right panel) in TDCC assay with FLT3-positive and FLT3-negative cell lines cultured at a 1:10 ratio with human pan T cells for 48 hours (mean ± SD, n=6 technical replicates, representative curves for one of ≥ three T cell donors). Expression of (B) CD69 and (C) CD25 on T cells from TDCC assays of FLT3-positive cell lines MOLM-13 and EOL-1 or FLT3-negative cell line A2780 co-cultured with human PBMCs at a 1:5 ratio with AMG 427 for 48 hours (mean ± SD, n=2 technical replicates; each curve represents a different PBMC donor). Concentration of (D) IFNγ and (E) TNFα in supernatants of TDCC assay of human PBMCs and EOL-1 cells (5:1 ratio) at time points indicated (mean ± SD, n=3 technical replicates of one representative donor). (F)TDCC of AMG 427 in the presence of sFLT3 (mean ± SD, n =2 technical replicates, 1 representative donor of 2). (G) Specific cytotoxicity of AMG 427 in the presence or absence of 10 ng/mL sFLT3L (mean ± SD, n = 3 technical replicates).

**Figure 4.** AMG 427 extends survival in mouse xenograft models. Survival analysis of (A) EOL-1 and (B) MOLM-13 orthotopic mouse models treated with AMG 427 or vehicle. Arrows indicate days of treatment. (n=5, vehicle group; n=10, all other groups). Statistical significance was determined using Kaplan-Meier estimator with Mantel-Cox logrank to compare treated
groups with the vehicle + T cells control group. (C) Pharmacokinetic profile of AMG 427 in mouse serum at times indicated after last administered dose in (B).

**Figure 5.** Preclinical PK and PD profile of FLT3 BiTE® molecules (experimental, A-D; HLE, E-G) in cynomolgus monkeys. (A) Serum concentration of experimental FLT3 BiTE® molecule in cynomolgus monkeys dosed by continuous intravenous infusion. Data for each animal plotted separately (n=3). Abundance of *FLT3* transcript in (B) bone marrow at day 17 and (C) blood measured by ddPCR at the times indicated dosed as in (A); percent decrease in *FLT3* transcript shown for each time point (each animal plotted separately, lines represent mean, n=3 technical replicates). (D) Serum concentration of sFLT3L for three different animals dosed as in (A); mean ± SD, n=1-3 technical replicates. (E) Mean exposure of AMG 427 ± SD (n=3/group) represented as area under the curve (AUC) in nanomolar times 7 days. Abundance of *FLT3* transcript measured by ddPCR at the times indicated in (F) bone marrow and (G) blood in animals dosed as in (A). Lines represent mean, n=3/group; percent decrease in *FLT3* transcript shown for each time point. (H) Serum concentration of sFLT3L in cynomolgus monkeys dosed as in (A); mean ± SD, n=3/group.

**Figure 6.** FLT3 BiTE® molecule cytotoxicity in AML patient samples *ex vivo* as a single agent and in combination with a PD-1–blocking antibody or TKIs. (A) Specific cytotoxicity relative to control BiTE® molecule for 14 primary AML patient samples cultured with experimental FLT3 BiTE® molecule for 9 days. Patients were grouped according to their responses: continued responders (green symbols, left panel), partial responders (green symbols, center panel) and non-responders (red symbols, right panel). (B) Initial MFI Ratio vs. autologous E:T cell ratio of 14 primary AML patients evaluated in (A). Vertical dotted line represents FLT3 positivity (MFI ≥ 1.5). Horizontal dotted line represents high E:T (75th percentile, 1:38). Green shading represents
E:T ratio and MFI with higher probability of showing response. (C) Specific cytotoxicity of parental (black circles) or PD-L1–transfected (red circles) MOLM-13 cells cultured for 24 hours 1:1 with CD3/CD28-activated human pan T cells and a dose range of AMG 427. Data are shown as mean ± SD (n=2 technical replicates, one of two representative T cell donors. (D) Specific cytotoxicity of PD-L1–transfected MOLM-13 cells cultured for 24 hours 1:1 with CD3/CD28-activated human pan T cells and AMG 427 with (orange squares) or without (black circles) 10 μg of an anti-PD-1 blocking antibody (mean ± SD, n=2 technical replicates, one of three representative T cell donors). (E) AMG 427 EC$_{50}$ ± anti-PD-1 blocking antibody as in (D), n=3 T cell donors, p = 0.02, paired t test.

**Figures**

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Figure 3

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Figure 6

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Time (Days)

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MFI Ratio FLT3

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Specific Cytotoxicity (%)

Log [AMG 427] (pM)

D

Specific Cytotoxicity (%)

Log [AMG 427] (pM)

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EC₅₀ (pM) AMG 427

Anti-PD-1 Antibody

p = 0.02
Molecular Cancer Therapeutics

Characterization of a Novel FLT3 BiTE® Antibody Construct for the Treatment of Acute Myeloid Leukemia

Bettina Brauchle, Rebecca L Goldstein, Christine M Karbowski, et al.

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