Therapeutic efficacy and safety of a human fusion construct targeting the TWEAK receptor Fn14 and containing a modified granzyme B

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ABSTRACT

Background Antibody-drug conjugates are an exceptional and useful therapeutic tool for multiple diseases, particularly for cancer treatment. We previously showed that the fusion of the serine protease granzyme B (GrB), the effector molecule of T and B cells, to a binding domain allows the controlled and effective delivery of the cytotoxic payload into the target cell. The production of these constructs induced the formation of high molecular aggregates with a potential impact on the efficacy and safety of the protein.

Methods Our laboratory designed a new Fn14 targeted fusion construct designated GrB(C210A)-Fc-IT4 which contains a modified GrB payload for improved protein production and preserved biological activity. We assessed the construct's enzymatic activity, as well as in vitro cytotoxicity and internalization into target cells. We also assessed pharmacokinetics, efficacy and toxicology parameters in vivo.

Results GrB(C210A)-Fc-IT4 protein exhibited high affinity and selective cytotoxicity within the nanomolar range when tested against a panel of Fn14-positive human cancer cell lines. The construct rapidly internalized into target cells, activating the caspase cascade and causing mitochondrial membrane depolarization. Pharmacokinetic studies in mice revealed that GrB(C210A)-Fc-IT4 displayed a bi-exponential clearance from plasma with a fast initial clearance ($t_{1/2\alpha}=0.36$ hour) followed by a prolonged terminal-phase plasma half-life ($t_{1/2\beta}=35$ hours). Mice bearing MDA-MB-231 orthotopic tumor xenografts treated with vehicle or GrB(C210A)-Fc-IT4 construct (QODx5) demonstrated tumor regression and long-term (>80 days) suppression of tumor growth. Treatment of mice bearing established, subcutaneous A549 lung tumors showed impressive, long-term tumor suppression compared with a control group treated with vehicle alone. Administration of GrB(C210A)-Fc-IT4 (100 mg/kg total dose) was well-tolerated by mice and resulted in significant reduction of tumor burden in a lung cancer patient-derived xenograft model. Toxicity studies revealed no statistically significant changes in aspartate transferase, alanine transferase or lactate dehydrogenase in treated mice. Histopathological analysis of tissues from treated mice did not demonstrate any specific drug-related changes.

Conclusion GrB(C210A)-Fc-IT4 demonstrated excellent, specific cytotoxicity in vitro and impressive in vivo efficacy with no significant toxicity in normal murine models. These studies show GrB(C210A)-Fc-IT4 is an excellent candidate for further preclinical development.

BACKGROUND

Despite the numerous and successful advances in cancer therapeutic strategies in the past few years, there is an unmet need of developing drugs with completely new mechanisms of action. Monoclonal antibody (mAb) therapeutics have now become standard treatment for different disease indications including cancer, inflammatory and autoimmune diseases.¹ More recently, antibody-drug conjugates (ADCs), which combine the cell binding specificity of mAbs with the cytotoxic potential of chemotherapeutic agents, have demonstrated significant clinical success. One of the most critical parameters in ADC design is selection of the antigen target because it has a direct impact on toxicity profile, efficacy and therapeutic window.^{2,3}

The tumor necrosis factor (TNF) superfamily cytokine TNF-related weak inducer of apoptosis (TWEAK) acts via a cell surface receptor named Fn14, and the TWEAK/Fn14 axis is an important molecular pathway involved in the regulation of tissue responses following acute injury.^{4–8} Overexpression of Fn14 or TWEAK has been documented in many solid tumor types including lung, breast and prostate^{9–14} and has been shown to be an independent negative prognostic indicator in a variety of tumor types.^{6,14–21} Therefore, interruption of the TWEAK/Fn14 signaling axis or the use of the external receptor domain to deliver specific cytotoxic agents appears to be avenues for targeted therapy development.^{8,22}

Previous studies have shown that anti-Fn14 antibodies are capable of suppressing the growth of tumor cells in vitro and in vivo.^{23–25} Clinical trials of antibodies targeting the TWEAK/Fn14 axis (drozitumab, enavatuzumab)^{26–28} have demonstrated safety and tolerability as well as anti-tumor effects in previously treated patients.

Our laboratory has previously reported that a human fusion protein construct targeting the Fn14 receptor and containing the cytotoxic granzyme B (GrB) payload (GrB-Fc-IT4) has in vitro and in vivo cytotoxic effects.⁷ During the course of our studies, however, we noticed the presence of high molecular aggregates during the production phase. We identified an unpaired Cys residue located in the C-terminal portion of GrB as a potential aggregation site. Here, we show the replacement of the impaired Cys had no impact on GrB enzymatic activity. In the present study we compared the in vitro cytotoxicity of GrB(C210A)-Fc-IT4 to GrB itself against a broad panel of non-small cell lung cancer (NSCLC) and other tumor cell lines. In addition, we conducted extensive mechanistic studies in vitro, as well as pharmacokinetic, toxicological and efficacy studies using several different in vivo tumor xenograft models.

METHODS

Cell lines

MDA-MB-453, MDA-MB-468, MDA-MB-231, MEF 3.5^{-/-}, A549, SKBR3, HT-29 and SKOV3 cell lines were obtained from the American Type Culture Collection and maintained as described previously described.²⁹

Plasmid construction, protein expression and purification

To generate the GrB(C210A)-Fc-IT4, human GrB(C210A) cDNA was fused to the N-terminus of the coding region of hinge CH2 and CH3 (Fc) of a human IgG1 heavy chain followed by the humanized single-chain VH-VL variable fragment of the anti-Fn14 (scFvIT4) antibody using three separate PCR steps. The individual PCR fragments were then subjected to two splice overlap extension PCR reactions (first Fc and scFvIT4; then GrB(C210A) with Fc-IT4) to complete the fusion gene construct.³⁰ The complete GrB(C210A)-Fc-IT4 fusion gene was then cloned into the mammalian expression vector pSecTag (Life Technologies, Waltham, Massachusetts, USA), expressed by transfection of HEK-293E cells and purified by immobilized metal affinity chromatography as previously described.⁷ The leader sequence containing the c-Myc epitope tag, (His)6 tag and the enterokinase cleavage site was cleaved by incubating the purified protein with recombinant enterokinase (New England Biolabs, Ipswich, Massachusetts, USA) overnight (20 U/mg) at room temperature (RT).³¹

GrB activity assay

GrB enzymatic activity was measured using a continuous colorimetric assay with Ac-IEPD-pNA

(N-acetyl-Ile-Glu-Pro-Asp-p-nitroanilide) (Merck, Darmstadt, Germany) as a specific substrate as previously described.^{7,32}

In vitro cytotoxicity assays

Cells were seeded onto plates overnight. After 24 hours incubation at 37°C, medium was replaced with complete media containing various concentrations of GrB or GrB(C210A)-Fc-IT4 and incubated at 37°C in 5% CO₂ for 72 hours. Cell viability was determined using the crystal violet staining method as described previously.³³

Internalization analysis

A549 and MDA-MB-231 cell lines were treated with 30 nmol/L GrB or GrB(C210A)-Fc-IT4 for various time periods. Internalization was assessed with an immunofluorescence assay as previously described.⁷ Studies to identify potential delivery of the GrB(C210A)-Fc-IT4 construct in the endosomal/lysosomal compartment were performed using the Lysotracker DND-22 kit (Life Technologies, Carlsbad, California, USA). A549 cells were treated with 30 nmol/L GrB(C210A)-Fc-IT4 labeled with Alexa Fluor 594 (Molecular Probes, Eugene, Oregon, USA) for 2 hours at 37°C, then media was replaced with fresh media containing the Lysotracker Blue DND-22 for 2 hours. Then cells were washed with phosphate buffered saline (PBS) and visualized with a Nikon A1Rsi multiphoton/confocal microscope using 405 nm and 561 nm lasers for excitation.

Apoptosis assays

Apoptosis was assessed using the Annexin V-FITC Kit (Invitrogen, Eugene, Oregon, USA).³⁴ Mitochondrial membrane depolarization was studied using the cationic dye JC-1 (MitoProbe JC-1 Assay Kit, ThermoFisher Scientific, Eugene, Oregon, USA) as previously described.³¹

Western blot analysis

Western blot studies were performed using standard procedures.⁷ The primary antibodies used were anti-GrB (Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-Caspase-3, anti-Caspase-7, anti-Caspase-9, anti-PARP-1 (Cell Signaling Technology, Danvers, Massachusetts, USA) and anti-β-actin (Sigma, St Louis, Missouri, USA).

Antibody dependent cell cytotoxicity assay

The antibody dependent cell cytotoxicity (ADCC) response was evaluated using the ADCC Reporter Bioassay kit (Promega, Madison, Wisconsin, USA). Briefly, SK-BR-3 cells were seeded onto plates and incubated overnight at 37°C. Serial dilutions of Trastuzumab (Roche, San Francisco, California, USA), an anti-CD20 antibody and GrB(C210A)-Fc-IT4 were prepared in RPMI-1640 media and added to SK-BR-3 cells. Jurkat/FcγRIIIa/NFAT-Luc cells were suspended in RPMI-1640 with low IgG serum and added to the cells, followed by incubation at 37°C. After 24 hours, luciferase activity was measured by ONE-Glo Luciferase Assay Reagent.

Figure 1 Schematic design, production and in vitro activity of the GrB(C210A)-Fc-IT4 protein. (A) Schematic diagram of the GrB(C210A)-Fc-IT4 construct. (B) SDS-PAGE analysis and Coomassie blue staining of purified GrB(C210A)-Fc-IT4 on a 6% gel run under reducing or non-reducing conditions. (C) GrB enzymatic activity. Purified GrB(C210A)-Fc-IT4 and GrB were incubated at 25°C with chromogenic substrate (Ac-IEPD-pNA) for the indicated times and absorbance was measured at 405 nm. His6, polyhistidine tag to facilitate purification; L-CH2-CH3, hinge and CH2 and CH3 domains of a human IgG1 heavy chain fragment; LS, the Igκ leader sequence that promotes the secretion of the fusion protein into the cell culture media; scFvIT4, humanized single chain fragment variable anti-Fn14 antibody.

In vitro stability of GrB(C210A)-Fc-IT4

GrB(C210A)-Fc-IT4 (50 µg/mL) was incubated with commercial mouse serum (BioIVT, Westbury, New York, USA) for different time periods (0, 24, 48, 72, 96 hours) at 37°C. Samples were harvested on the same day and spun through microtainer vials. The anti-GrBhorseradish peroxidase (HRP) conjugated antibody (Santa Cruz Biotechnology) was used to detect the target protein and the signal intensity was quantitated by Adobe Photoshop with GrB(C210A)-Fc-IT4 as standard. The signal was normalized with a serum loading control (Transferrin, Genetex, Irvine, California, USA). Stability experiments were performed in duplicate.

Pharmacokinetic studies

BALB/c mice (Experimental Radiation Oncology Breeding Core, MD Anderson Cancer Center) at 4–6 weeks of age were injected (intravenous, tail vein) with 200 µg GrB(C210A)-Fc-IT4. At various time points (0–48 hours after fusion protein administration), mice were sacrificed and blood was extracted from the chest cavity. Plasma samples were analyzed using the Western blot

method previously described as well as a quantitative ELISA assay. Briefly, ELISA microtiter plates (Santa Cruz Biotech) were coated (18 hours, 4°C) with 2 µg/mL of the Fn14 extracellular domain (Cell Signaling) in coating buffer (0.05 M Na₂CO₃, 0.05 M NaHCO₃, pH 9.6) and blocked with 2% bovine serum albumin in PBS at RT for 2 hours. Samples (100 µL) were loaded in triplicate and incubated for 2 hours at RT, followed by the addition of HRP-conjugated goat anti-human IgG (KLP, Gaithersburg, Maryland, USA) in blocking buffer (1 hour, RT). Plates were washed five times after each step. The reaction was visualized by the addition of chromogenic substrate (ABTS) for 30 min and stopped with 10% sodium dodecyl sulfate (SDS). Absorbance at 450 nm was measured on a Thermomax plate reader. As a reference for quantification, a standard curve was established by a serial dilution of recombinant GrB(C210A)-Fc-IT4 in commercial mouse serum. Pharmacokinetic parameters were calculated by compartmental analysis of plasma data using an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel the PKSolver (V.2.0).

Table 1 Comparative IC₅₀ values of GrB and GrB(C210A)-Fc-IT4 against various cancer cell lines

Cell line	Tumor type	Fn14 levels	IC ₅₀ (nmol/L)	
			GrB*	GrB(C210A)-Fc-IT4*
MDA-MB-231	Breast	++++	>1700	6
MDA-MB-453	Breast	++	>1400	7
MDA-MB-468	Breast	+++	>900	14
MCF7	Breast	++	>700	15
SK-BR3	Breast	+	>700	65
HT29	Colon	++	>700	50
A549	Lung	++	601	25
SKOV3	Ovarian	++++	>700	13
MEF 3.5 ^{-/-}	Mouse embryonic fibroblast	–	>700	>580

*Mean of at least three independent experiments.
GrB, granzyme B.

In vivo efficacy using nude mice bearing human breast or lung cancer tumor xenografts

Groups of female BALB/c nude mice (MDACC Experimental Radiation Oncology Breeding Core) (7 weeks old, five mice per group) were injected with 5×10^6 human MDA-MD-231/Luc cells into the mammary fat pad (1:1 v/v with Matrigel). Once tumor volumes reached between 40 and 60 mm³, animals were randomized and treated (iv, tail vein) every other day for five total injections with vehicle or GrB(C210A)-Fc-IT4 (100 mg/kg total dose). Animals were monitored and tumor growth was measured every 3–4 days for an additional 90 days.

Human A549 lung cancer cells (7×10^6 cells) were subcutaneously injected on the right flank of 6-week-old female infant BALB/c nude mice. Once tumors were established (~25 mm³), animals were randomized and treated (intra-peritoneal) every day for 10 total injections with vehicle, enavatumumab (50 mg/kg) or GrB(C210A)-Fc-IT4 (50 mg/kg or 100 mg/kg total dose). Animals were monitored and tumor growth was measured every 3–4 days for 60 days.

In vivo efficacy study using lung cancer patient-derived xenograft model

Animal experiments were conducted following approval by the Institutional Animal Care and Use Committee guidelines of St. Joseph's Hospital and Medical Center. Fresh lung tumors resected from a patient-derived xenograft (PDX) were subcutaneously (s.c.) implanted into the flank of 6-week-old nu/nu athymic mice (Jackson Labs). Tumors from the same patient xenografts were allowed to grow to approximately 200 mm³. Animals were grouped randomly (six mice per group) and treated with vehicle or GrB(C210A)-Fc-IT4 (20 mg/kg) every other day over a 10-day period for a total dose of 100 mg/kg via tail vein injection. Tumor size was measured 2–3 times per week for 30 days.

Toxicology studies

Female BALB/c mice (4–6 weeks old) were injected intravenously every other day for five total injections of vehicle or GrB(C210A)-Fc-IT4 (100 mg/kg total dose). One day after the last injection (Acute group, Day 10) or 4 weeks after the last injection (Recovery group, Day 38) animals in the study were euthanized and terminally bled for hematological and tissue harvest/histological analyzes. The studies were performed by the MDACC Department of Veterinary Medicine and Surgery.

Statistical analysis

All in vivo efficacy experiments, statistical analysis was performed using Prism V.8.0.0 (GraphPad Software). Comparisons of continuous variables between two groups were performed using unpaired t-test. Comparisons of continuous variables between three or more groups were performed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Differences were considered to be statistically significant when p values were <0.05.

RESULTS

Construction, expression and purification of GrB(C210A)-Fc-IT4 fusion protein

GrB(C210A)-Fc-IT4 (figure 1A) was transiently expressed by HEK-293E cells and purified to homogeneity. Analysis by SDS-PAGE of GrB(C210A)-Fc-IT4 under non-reducing conditions confirmed the purity of the construct (figure 1B). The theoretical molecular weight is 160 kDa for the homodimer but the presence of several glycosylation sites in the primary sequence increased the apparent molecular weight to 220 kDa. GrB(C210A)-Fc-IT4 had a similar enzymatic activity to commercial native GrB (264 U/nmol and 159 U/nmol, respectively), demonstrating the mutation has no impact on the enzymatic activity of GrB (figure 1C).

Cytotoxic effect of GrB(C210A)-Fc-IT4 against various tumor cell lines and correlation with exposure time

Next, we assessed the cytotoxic effects of GrB(C210A)-Fc-IT4 on a panel of tumor cell lines (table 1). The IC_{50} of GrB(C210A)-Fc-IT4 ranged in the low nanomolar range, showing that the construct is able to induce cell death on Fn14 positive cell lines in a targeted manner. As previously published for the original GrB-Fc-IT4,⁷ we found no correlation between cell surface Fn14 levels and the sensitivity to the construct (data not shown). The fusion protein did not affect the growth of the mouse embryonic fibroblast MEF3.5^{-/-} cell line, derived from Fn14 Knockout mice, indicating that the cytotoxicity of the construct on the tumor cell lines was dependent on the presence of the Fn14 receptor.

We also examined the effect of GrB(C210A)-Fc-IT4 on MDA-MB-231 viability over time. While GrB(C210A)-Fc-IT4 treatment for 12 hours showed moderate levels of cytotoxicity, the lowest IC_{50} was obtained after 72 hours of continuous exposure (online supplemental table 1S).

These results suggested cytotoxicity was due to receptor-mediated internalization and subsequent release of GrB(C210A)-Fc-IT4 into the cytosol.

Internalization and intracellular localization of GrB(C210A)-Fc-IT4

To investigate the cytotoxic effect of the GrB(C210A)-Fc-IT4 on Fn14 positive cells, we first assessed the internalization process in MDA-MB-231 and A549 cells by immunofluorescence microscopy. We detected GrB(C210A)-Fc-IT4 in the cytoplasm within 30 min after exposure (figure 2A). In contrast, no staining was detected in cells treated with GrB alone, suggesting that internalization of GrB(C210A)-Fc-IT4 was driven by Fn14 receptor-mediated endocytosis. The intracellular delivery of the GrB portion and the metabolic fate of GrB(C210A)-Fc-IT4 was assessed at various times by Western blot analysis. Intact GrB(C210A)-Fc-IT4 was detected within the cell in less than 1 hour after exposure (figure 2B). Intracellular cleavage to release free GrB into the cytosol was

Figure 2 Internalization and localization of GrB(C210A)-Fc-IT4. (A) MDA-MB-231 and A549 cells were treated with 30 nmol/L GrB or GrB(C210A)-Fc-IT4 for the indicated times. Cells were acid washed to remove surface bound material, fixed with phosphate buffered saline+3.7% paraformaldehyde, immunostained for the presence of GrB (green) and were counterstained with propidium iodide to identify nuclei (red). (B) Western blot analysis of the intracellular fate of 25 nmol/L GrB(C210A)-Fc-IT4 added to MDA-MB-231 cells positive controls are shown on the right side. (C) MDA-MB-231 cells were treated with different concentrations of GrB(C210A)-Fc-IT4 in the presence (25 μmol/L □; 10 μmol/L △) or absence (●) of the lysosomal inhibitor chloroquine and cell viability was measured at 72 hours. Cell viability was plotted as mean±SD of % of control. (D) Intracellular localization of GrB(C210A)-Fc-IT4. A549 cells were incubated for 2 hours with 30 nM Alexa-549-GrB(C210A)-Fc-IT4 (red) followed by addition of Lysotracker DND22 (blue). Cells were imaged live by confocal microscopy. Magnification 20×.

observed at approximately 4 hours of treatment. Surprisingly, neither the intact GrB(C210A)-Fc-IT4 nor free GrB were observed after 16 hours, suggesting either complete degradation of the construct and its components or epitope masking of the GrB portion of the molecule.

Because accumulation of cell-targeted fusion proteins can occur in the endosomal and lysosomal compartments and can result in significant reduction in the efficiency of certain targeted therapeutics, we next evaluated the impact of chloroquine, a lysosomotropic agent that allows endosomal escape, on construct activity against MDA-MB-231 cells. Treatment of cells with different (non-cytotoxic) concentrations of chloroquine had no impact on the IC₅₀ of GrB(C210A)-Fc-IT4 suggesting that endosomal or lysosomal trapping does not play a role in GrB(C210A)-Fc-IT4 internalization (figure 2C). We also studied the localization of GrB(C210A)-Fc-IT4 protein within the endosomes or lysosomes of A549 cells using LysoTracker Blue DND-22, a fluorescent dye that stains acidic compartments in live cells. We did not detect specific colocalization of the fusion protein in endosomes or lysosomes after 2 hours of treatment (figure 2D). Together, these studies confirm that GrB(C210A)-Fc-IT4 is internalized rapidly and efficiently escapes into the cytosol of target cells without appreciable accumulation in the lysosomal or endosomal compartments.

Mechanism of GrB(C210A)-Fc-IT4 induced apoptosis

GrB is a well-known potent initiator of apoptosis once delivered to the cytosol.³⁵ To confirm that the cytotoxicity of GrB(C210A)-Fc-IT4 is mediated by apoptosis, we first assessed the Annexin V levels on MDA-MB-231 and A549 cell lines in the presence of different concentrations of either GrB(C210A)-Fc-IT4 or GrB. The construct GrB(C210A)-Fc-IT4 was able to increase Annexin V levels whereas GrB treatment alone showed no impact over 24 hours compared with non-treated cells (figure 3A). Similarly, treatment with GrB(C210A)-Fc-IT4 rapidly induced mitochondrial depolarization in treated cells over 24 hours (figure 3B).

Next, we used Western blot analysis to examine various cellular mediators of apoptosis known to be part of the GrB-induced apoptotic mechanism. Cells treated with GrB(C210A)-Fc-IT4 showed activation of the apoptotic cascade by cleavage of caspase-3, caspase-7, caspase-9 and PARP-1 at 8 hours after treatment (figure 3C). These data confirm that the mechanism of cytotoxic action appears to be similar to that of GrB itself.

An important mechanism of cytotoxic action of some conventional IgG surface-bound antibodies is the mediation of ADCC. To evaluate whether the Fc portion of the GrB(C210A)-Fc-IT4 is able to mediate ADCC on target cells, we performed an in vitro assay using the SK-BR-3 cell line which is known to express Fn14 and HER2 on the cell surface.³⁶ As shown in figure 3D, treatment with the anti-HER2 antibody, trastuzumab, induced the activation of Jurkat cells whereas treatment with GrB(C210A)-Fc-IT4 and the irrelevant control antibody did not induce

any response in the effector cells. This suggests that GrB(C210A)-Fc-IT4 is unable to induce ADCC likely due to the steric hindrance at the C-terminus of the Fc domain by the presence of the anti-Fn14 scFv domain. Therefore, all of the cytotoxic effects observed in vivo are likely to be solely due to the GrB component of the fusion construct.

Ex vivo stability and pharmacokinetics of GrB(C210A)-Fc-IT4

In order to assess the stability of the GrB(C210A)-Fc-IT4 molecule in a physiological environment, we incubated GrB(C210A)-Fc-IT4 in commercial mouse serum for up to 96 hours at 37°C and conducted Western blot analysis. Intact or full length GrB(C210A)-Fc-IT4 was present in serum for up to 96 hours with an overall ex vivo half-life of 88 hours, indicating the construct is stable under physiological conditions (figure 4A). In addition, quantitative ELISA and Western blot analysis of GrB(C210A)-Fc-IT4 from plasma samples harvested at 24, 48 and 72 hours after administration to BALB/c mice showed similar quantitative results (data not shown).

Pharmacokinetic analysis of GrB(C210A)-Fc-IT4 revealed a biphasic clearance profile (figure 4B). Following intravenous administration, GrB(C210A)-Fc-IT4 demonstrated a rapid α -phase half-life of 0.36 hour, followed by a prolonged terminal β -phase clearance of 35.6 hours. The apparent small volume of distribution of 1.66 mL suggests limited distribution of GrB(C210A)-Fc-IT4 into peripheral sites outside the vascular space immediately after administration. The estimated initial (t=0) concentration in plasma was 120 μ g/mL, similar to the projected initial dose concentration of 153 μ g/mL of drug injected per mouse. These studies further confirm the stability of the molecule in vivo.

Toxicity studies of GrB(C210A)-Fc-IT4 in BALB/c mice

Next, we examined the in vivo toxicity observed in mice treated with total dose of 100 mg/kg of GrB(C210A)-Fc-IT4 using a QODX5 schedule. Body weight, tissue and serum samples were collected 24 hours (acute group) or 4 weeks (recovery group) after intravenous administration of the construct to BALB/c mice. There were no differences in body weight (figure 4C) or specific organ weight (data not shown) between the controls and the treated mice. The enzymatic activities of liver enzymes were determined and no differences in aminotransferase (AST), alanine transferase (ALT) and lactate dehydrogenase (LDH) levels between groups were found, indicating no evidence of hepatotoxicity (figure 4D). We performed a complete hematological and chemical analysis; we did not find significant differences between groups (data not shown). The histological analysis showed no moderate or marked lesions in any mice, and only a few rare and minor mild incidental lesions were present (online supplemental table 2S). Thus, GrB(C210A)-Fc-IT4 is a safe and well-tolerated drug at doses as high as 100 mg/kg in mice as there were no indications of severe toxicity.

Figure 3 Mechanism of action of GrB(C210A)-Fc-IT4 fusion construct. (A) MDA-MB-231 and A549 cells were either left untreated or treated with the indicated agents at the indicated concentrations for 48 hours. Cells were then stained with Alexa Fluor 488 Annexin V and propidium iodide, followed by flow cytometric analysis. The percentage of annexin V⁺ cells includes early (Annexin V⁺ PI⁻) and late (Annexin V⁺ PI⁺) apoptosis. Vinblastine was used as a positive control for apoptosis. (B) MDA-MB-231 and A549 cells were either left untreated or treated and then assayed for mitochondrial membrane depolarization by flow cytometry. (C) MDA-MB-231 and A549 cells were left untreated or treated with 100 nmol/L GrB(C210A)-Fc-IT4 for the indicated times. Cells were collected and whole-lysates were analyzed by Western blot with the indicated antibodies. (D) SK-BR-3 cells were either left untreated or treated with GrB(C210A)-Fc-IT4, trastuzumab or a negative control antibody anti-CD20 at different concentrations. Antibody dependent cell cytotoxicity bioassay effector cells were added and after 24 hours induction at 37°C, Bio-Glo luciferase assay reagent was added and luminescence determined using a GloMax-Multi+Luminometer. The data were plotted as mean±SD and fitted to a 4PL curve using GraphPad Prism software.

In vivo efficacy of GrB(C210A)-Fc-IT4 in xenograft models

Once the pharmacokinetics and toxicity of GrB(C210A)-Fc-IT4 were determined, we then evaluated the ability of GrB(C210A)-Fc-IT4 to inhibit the growth of established MDA-MB-231 tumors in BALB/c nude mice. Three out of five mice with tumors treated every other day with GrB(C210A)-Fc-IT4 for 10 days showed complete tumor growth inhibition whereas the other two mice showed a significant tumor growth inhibition ($p < 0.001$) compared with control (vehicle) tumors 80 days after implantation (figure 5A). Furthermore, mice bearing MDA-MB-231 tumors treated via intravenous or intraperitoneal injection with GrB(C210A)-Fc-IT4 (100 mg/kg) following the same scheduled (QODX5) showed significant tumor growth delay independent of the route of administration (online supplemental figure 1S). There were no changes in body weight in control versus treated mice for the

duration of both studies indicating that 100 mg/kg total dose was well tolerated in mice.

In order to determine whether the antitumor effect observed was mediated by the cytotoxic payload (GrB) and not because of TWEAK/Fn14 axis inhibition by the scFvIT4 targeting moiety, we treated nude mice with subcutaneous A549 NSCLC tumors every day for 10 days with either GrB(C210A)-Fc-IT4 or the unconjugated anti-Fn14 antibody, enavatuzumab at equimolar drug concentrations.^{27 28} Administration of enavatuzumab resulted in transient tumor growth inhibition followed by rapid regrowth of the tumors back to and exceeding control tumor growth (figure 5B). In contrast, treatment with our GrB-based construct was able to induce a sustained prolonged growth delayed when compared with controls.

Finally, we examined the efficacy of GrB(C210A)-Fc-IT4 in a human lung PDX model. Tumor cells were

Figure 4 Stability, pharmacokinetics and toxicology of GrB(C210A)-Fc-IT4 in mice. (A) GrB(C210A)-Fc-IT4 was incubated in mouse serum (50 $\mu\text{g/mL}$) at 37°C for various lengths of time and the presence of intact GrB(C210A)-Fc-IT4 was assessed by Western blot analysis. Estimated percentage of the initial material was plotted as mean $\pm$ SD versus time to obtain the ex vivo half-life. (B) GrB(C210A)-Fc-IT4 was injected via tail vein in BALB/c mice. Groups of mice (three mice per group) were sacrifice 0, 5, 15, 20 min, 1, 2, 4, 8, 16, 24 and 48 hours after injection. Heparinized blood samples were harvested from the chest cavity and centrifuged, the plasma fraction was analyzed for the presence of fusion protein using a GrB capture ELISA method. Pharmacokinetic parameters were calculated by compartmental analysis of plasma data using the PKSolver add-in program (V.2.0). $t_{1/2\alpha}$ and $t_{1/2\beta}$ represent half-lives in the initial distribution phase and terminal elimination phase, respectively; (C) Body weight in vehicle group (phosphate buffered saline 10% Maltose) and GrB(C210A)-Fc-IT4 (100 mg/kg) treated mice. The values represent the weights measured in BALB/c mice before treatment, one after treatment (acute group) and 4 weeks after treatment (recovery group). (D) Liver enzymatic activity detected in serum of BALB/c treated mice. The enzymatic activity of ALT, AST and LDH was measured in acute and recovery groups. No statistical difference was found between mice treated with vehicle or GrB(C210A)-Fc-IT4 neither in the acute or the recovery groups. $AUC_{0-\infty}$, area under the blood concentration versus time curve; C_0 , estimated initial drug concentration in the blood; CL, total body clearance; MRT, mean resident time; V_d , volume of distribution of the central compartment; V_{ss} , volume of distribution at steady state.

subcutaneously (s.c.) implanted in the flank of mice and once tumors reached about 200 mm³, animals were grouped randomly (six mice per group) and treated with PBS or GrB(C210A)-Fc-IT4 (100 mg/kg). We observed a 50% reduction in tumor burden in GrB(C210A)-Fc-IT4 treated mice compared with the vehicle group (figure 5C). Overall, these data indicate that GrB(C210A)-Fc-IT4 is effective against breast and lung tumor xenograft models causing a dramatic and prolonged tumor growth inhibition, and also reduces tumor growth rate in a lung PDX tumor model.

DISCUSSION

Cancer treatment has been evolving over the years, from basic strategies including surgery, radiation and systemic chemotherapy to more elaborate targeted approaches such as immunotherapy and personalized molecularly targeted medicine. In recent years, the use of chemotherapy has been augmented thanks to the development of targeted therapies like kinase inhibitors, ADCs or similar molecules.³⁷ We have previously demonstrated that the GrB-based fusion construct targeting Fn14 is an effective and powerful therapeutic.⁷ In this study we extended these findings and demonstrated that the

specific mutation introduced into the new GrB(C210A)-Fc-IT4 fusion construct did not impact the potent cytotoxic effect of the drug against a large panel of tumors cells. GrB(C210A)-Fc-IT4 is able to internalize into Fn14 positive cells in a rapid and specific manner via endocytosis and once it is in the cytoplasm it induces cell death via the GrB portion of the construct.

GrB is a highly cytotoxic serine protease expressed by human immune effector cells and is the causative agent whereby these cells induce pro-apoptotic signals when delivered to the cytoplasm of target cells.³⁸ The protein has been well studied and is known to operate through multimodal cascade pathways, which are both caspase dependent and independent. We have previously shown that GrB-based fusion constructs mechanism of action includes activation of the caspase cascade and release of cytochrome c from the mitochondrial compartment⁷ resulting in activation of a caspase-independent apoptotic cascade exemplified by mitochondrial depolarization. The well-known trimodal mechanism of GrB cytotoxic activity is different from that of the small cytotoxic molecules used in other ADCs such as auristatin or maytansine derivatives which are microtubule inhibitors.³⁹ Importantly, while intrinsic resistance mechanisms

Figure 5 In vivo activity of the GrB(C210A)-Fc-IT4 fusion construct on different tumor models. (A) MDA-MB-231-Luc cells were implanted under the mammary fat pad of nude mice and groups of mice (n=5) were treated intravenous with vehicle (phosphate buffered saline (PBS) 10% maltose●) or GrB(C210A)-Fc-IT4 (100 mg/kg□). Treatment was every other day when the tumors reached approximately 75 mm³, arrows represent treatment day. Tumor size was assessed by direct caliper measurement. Efficacy data were plotted as mean tumor volume (in mm³)±SD. ***p<0.0003, compared with vehicle group. (B) A549 cells were implanted subcutaneously on the right flank of nude mice and groups of mice (n=5) were treated intraperitoneal with vehicle (PBS 10% maltose●), enavatuzumab (50 mg/kg□) or GrB(C210A)-Fc-IT4 (50 mg/kg △, 100 mg/kg□). Treatment was every day (10 doses) when the tumors reached approximately 25 mm³. Arrows represent treatment day. Tumor size was assessed by direct caliper measurement. Efficacy data were plotted as mean tumor volume (in mm³)±SD. *p<0.0382, **p<0.0337, compared with vehicle group. (C) Fresh tumor cells from a patient were implanted subcutaneously (s.c.) into the flanks of 6-week-old nude mice, when the tumors reached approximately 200 mm³ animals were grouped randomly (n=6) and treated every other day intravenous route with vehicle (PBS●), or GrB(C210A)-Fc-IT4 (100 mg/kg□). **p<0.0042, compared with vehicle. Efficacy data were plotted as mean tumor volume (in mm³)±SD. Treatment days are indicated with arrows.

such as multidrug resistance (MDR) drug efflux pumps are known to confer resistance to apoptosis, previous studies showed that expression of MDR did not result in resistance mechanisms to GrB-based targeted fusion constructs like GrB(C210A)-Fc-IT4.⁷

The cell surface receptor Fn14 for the cytokine TWEAK has emerged as a valuable target for cancer therapy because it displays low expression in most normal tissues and has been shown to be significantly elevated in a variety of solid tumor types.^{7 9-13 40 41} Previously we were not able to find a correlation between the level of Fn14 overexpression in cancer cells and sensitivity to GrB(C210A)-Fc-IT4. We have shown similar results with other GrB-based constructs targeting different molecules like HER2 or VEGFR2.^{7 31 42} These data suggest that target abundance alone does not determine sensitivity. Other mechanisms such as target availability, endocytosis, endosomal release or apoptosis induction by GrB may play a determining factor. We are currently performing additional studies to explore more closely the intracellular elements related to the signaling pathways involved in the GrB cytotoxic mechanism(s) of action.

An important parameter in the development of antibody drug conjugates is pharmacokinetics studies to

provide critical information about drug's behavior and metabolism in vivo. In our ex vivo studies we were able to demonstrate intact GrB(C210A)-Fc-IT4 up to 96 hours in circulation. We were unable to analyze for specific GrB enzymatic activity, because endogenous levels of free GrB present in the serum would interfere with the assay. The pharmacokinetic analysis showed that GrB(C210A)-Fc-IT4 has a secondary half-life over 35 hours in BALB/c mice, with a plasma concentration over 10 µg/mL at 48 hours after injection. Although GrB(C210A)-Fc-IT4 has a fast initial clearance, it is highly stable in circulation, which corresponds with bi-exponential kinetics. As expected, in our design of the construct, the dimeric molecular weight of GrB(C210A)-Fc-IT4 offered a prolonged circulation in blood of mice, which potentially allows higher accumulation within a tumor. It is important to note that the original anti-Fn14 antibody we employed to generate the IT4 scFv (ITEM4) cross-reacts with the murine Fn14 receptor.⁴³ Therefore, toxicity in normal murine tissues observed with GrB(C210A)-Fc-IT4 will be important indicators to consider for human clinical trials. In addition, other studies have shown that human GrB is approximately 30-fold more toxic than murine GrB.⁴⁴ Therefore, toxicity (if any) observed by the GrB(C210A)-Fc-IT4

construct studies in mice may over predict for clinical toxicity. Our toxicity studies performed in BALB/c mice without tumors showed that GrB(C210A)-Fc-IT4 is a safe and tolerable drug at doses as high as 100 mg/kg. Moreover, we did not find signs of hepatotoxicity in the clinical chemistry or the histological analyzes. This is a compelling result as compared with other anti-Fn14 antibodies, like enavatuzumab, which induced mild elevations of the liver enzymes (ALT, AST) in their preclinical studies in non-human primates at the same dose.²⁸ Because of the absence of toxicity at the doses tested, we have yet to establish a maximum tolerated dose (MTD) for GrB(C210A)-Fc-IT4. Studies suggest that the MTD is likely above the efficacious 100 mg/kg total dose level employed in our studies.

The efficacy studies against MDA-MB-231 and A549 tumor xenografts and lung PDX tumor models demonstrated excellent in vivo tumor growth suppression. In addition, no significant difference was observed in the efficacy of mice treated via intravenous or intraperitoneal injection. The in vivo efficacy comparison between GrB(C210A)-Fc-IT4 and enavatuzumab in the A549 xenograft tumor model demonstrated that GrB plays a critical role in mediating the cytotoxicity with a negligible role played by the targeting moiety. All these results together demonstrate that GrB(C210A)-Fc-IT4 is an effective anti-tumor agent that should be considered for clinical evaluation in patients with Fn14-positive tumors.

Correction notice This article has been corrected since it was published. The author name 'Walter N Hittelman' was incorrectly spelt as 'Walter N Hittlerman'.

Contributors Conception and study design: AAdC, LHC, KAM and MGR. Development of methodology: AAdC, LHC, TGW, JAW, WNH, KAM and MGR. Data acquisition: AAdC, LHC, TGW, JAW, WNH, KAM and MGR. Analysis and interpretation of data: AAdC, LHC, TGW, WNH, KAM and MGR. Manuscript preparation: AAdC, LHC, WNH, KAM, JAW, TGW and MGR. Study supervision: AAdC and MGR. All authors read and approved the final manuscript.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information. Data are available upon reasonable request.

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REFERENCES

- Weiner GJ. Building better monoclonal antibody-based therapeutics. *Nat Rev Cancer* 2015;15:361–70.
- Lambert JM, Berkenblit A. Antibody-Drug conjugates for cancer treatment. *Annu Rev Med* 2018;69:191–207.
- Birrer MJ, Moore KN, Betella I, et al. Antibody-Drug Conjugate-Based therapeutics: state of the science. *J Natl Cancer Inst* 2019;111:538–49.
- Novoyatleva T, Sajjad A, Engel FB. Tweak-Fn14 Cytokine-Receptor axis: a new player of myocardial remodeling and cardiac failure. *Front Immunol* 2014;5:50.
- Burkly LC. Tweak/Fn14 axis: the current paradigm of tissue injury-inducible function in the midst of complexities. *Semin Immunol* 2014;26:229–36.
- Winkles JA. The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. *Nat Rev Drug Discov* 2008;7:411–25.
- Zhou H, Mohamedali KA, Gonzalez-Angulo AM, et al. Development of human serine protease-based therapeutics targeting Fn14 and identification of Fn14 as a new target overexpressed in TNBC. *Mol Cancer Ther* 2014;13:2688–705.
- Cheng E, Armstrong CL, Galisteo R, et al. Tweak/Fn14 Axis-Targeted therapeutics: moving basic science discoveries to the clinic. *Front Immunol* 2013;4:473.
- Hu G, Zeng W, Xia Y. Tweak/Fn14 signaling in tumors. *Tumour Biol* 2017;39:101042831771462.
- Wang X, Zeng Y, Ho DN, et al. Fibroblast growth factor-inducible 14: multiple roles in tumor metastasis. *Curr Mol Med* 2015;15:892–904.
- Li X, Zhu W, Chen Z, et al. Fibroblast growth factor-inducible 14 regulates cell growth and multidrug resistance of small-cell lung cancer through the nuclear factor-κB pathway. *Anticancer Drugs* 2014;25:1152–64.
- Yin J, Liu Y-N, Tillman H, et al. AR-regulated TWEAK-Fn14 pathway promotes prostate cancer bone metastasis. *Cancer Res* 2014;74:4306–17.
- Whitsett TG, Fortin Ensign SP, Dhruv HD, et al. Fn14 expression correlates with Met in NSCLC and promotes MET-driven cell invasion. *Clin Exp Metastasis* 2014;31: :613–23.
- Willis AL, Tran NL, Chatigny JM, et al. The fibroblast growth factor-inducible 14 receptor is highly expressed in HER2-positive breast tumors and regulates breast cancer cell invasive capacity. *Mol Cancer Res* 2008;6:725–34.
- Winkles JA, Tran NL, Brown SAN, et al. Role of TWEAK and Fn14 in tumor biology. *Front Biosci* 2007;12:2761–71.
- Sun Y, Han Y, Wang X, et al. Correlation of EGFR Del 19 with Fn14/JAK/STAT signaling molecules in non-small cell lung cancer. *Oncol Rep* 2016;36:1030–40.
- Meulendijks D, Lassen UN, Siu LL, et al. Exposure and tumor Fn14 expression as determinants of pharmacodynamics of the Anti-TWEAK monoclonal antibody RG7212 in patients with Fn14-Positive solid tumors. *Clin Cancer Res* 2016;22:858–67.
- Whitsett TG, Mathews IT, Cardone MH, et al. Mcl-1 mediates TWEAK/Fn14-induced non-small cell lung cancer survival and therapeutic response. *Mol Cancer Res* 2014;12:550–9.
- Kwon O-H, Park S-J, Kang T-W, et al. Elevated fibroblast growth factor-inducible 14 expression promotes gastric cancer growth via nuclear factor-κB and is associated with poor patient outcome. *Cancer Lett* 2012;314:73–81.
- Hersh DS, Harder BG, Roos A, et al. The TNF receptor family member Fn14 is highly expressed in recurrent glioblastoma and in GBM patient-derived xenografts with acquired temozolomide resistance. *Neuro Oncol* 2018;20:1321–30.
- Yang J, Min K-W, Kim D-H, et al. High TNFRSF12A level associated with MMP-9 overexpression is linked to poor prognosis in breast cancer: gene set enrichment analysis and validation in large-scale cohorts. *PLoS One* 2018;13:e0202113.
- Wajant H. The TWEAK-Fn14 system as a potential drug target. *Br J Pharmacol* 2013;170:748–64.
- Michaelson JS, Amatucci A, Kelly R, et al. Development of an Fn14 agonistic antibody as an anti-tumor agent. *MAbs* 2011;3:362–75.
- Michaelson JS, Kelly R, Yang L, et al. The anti-Fn14 antibody B1B036 inhibits tumor growth in xenografts and patient derived primary tumor models and enhances efficacy of chemotherapeutic agents in multiple xenograft models. *Cancer Biol Ther* 2012;13:812–21.

- 25 Trebing J, Lang I, Chopra M, *et al.* A novel llama antibody targeting Fn14 exhibits anti-metastatic activity in vivo. *MAbs* 2014;6:297–308.
- 26 Lassen UN, Meulendijks D, Siu LL, *et al.* A phase I monotherapy study of RG7212, a first-in-class monoclonal antibody targeting TWEAK signaling in patients with advanced cancers. *Clin Cancer Res* 2015;21:258–66.
- 27 Ye S, Fox MI, Belmar NA, *et al.* Enavatuzumab, a humanized Anti-TWEAK receptor monoclonal antibody, exerts antitumor activity through attracting and activating innate immune effector cells. *J Immunol Res* 2017;2017:1–14.
- 28 Lam ET, Eckhardt SG, Messersmith W, *et al.* Phase I study of Enavatuzumab, a first-in-class humanized monoclonal antibody targeting the TWEAK receptor, in patients with advanced solid tumors. *Mol Cancer Ther* 2018;17:215–21.
- 29 Cheung LH, Zhao Y, Alvarez-Cienfuegos A, *et al.* Development of a human immuno-oncology therapeutic agent targeting HER2: targeted delivery of granzyme B. *J Exp Clin Cancer Res* 2019;38:332.
- 30 Zhou H, Ekmekcioglu S, Marks JW, *et al.* The TWEAK receptor Fn14 is a therapeutic target in melanoma: immunotoxins targeting Fn14 receptor for malignant melanoma treatment. *J Invest Dermatol* 2013;133:1052–62.
- 31 Mohamedali KA, Cao Y, Cheung LH, *et al.* The functionalized human serine protease granzyme B/VEGF₁₂₁ targets tumor vasculature and ablates tumor growth. *Mol Cancer Ther* 2013;12:2055–66.
- 32 Ewen C, Kane KP, Shostak I, *et al.* A novel cytotoxicity assay to evaluate antigen-specific CTL responses using a colorimetric substrate for granzyme B. *J Immunol Methods* 2003;276:89–101.
- 33 Zhou H, Marks JW, Hittelman WN, *et al.* Development and characterization of a potent immunoconjugate targeting the Fn14 receptor on solid tumor cells. *Mol Cancer Ther* 2011;10:1276–88.
- 34 Geft D, Schwartzberg S, Rogowsky O, *et al.* Circulating apoptotic progenitor cells in patients with congestive heart failure. *PLoS One* 2008;3:e3238.
- 35 Goping IS, Barry M, Liston P, *et al.* Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. *Immunity* 2003;18:355–65.
- 36 Chao DT, Su M, Tanlimco S, *et al.* Expression of TweakR in breast cancer and preclinical activity of enavatuzumab, a humanized anti-TweakR mAb. *J Cancer Res Clin Oncol* 2013;139:315–25.
- 37 Nakashima L. Evolution of cancer treatment and evolving challenges. *Healthc Manage Forum* 2018;31:26–8.
- 38 Rousalova I, Krepela E. Granzyme B-induced apoptosis in cancer cells and its regulation (review). *Int J Oncol* 2010;37:1361–78.
- 39 Fu Y, Ho M. Dna damaging agent-based antibody-drug conjugates for cancer therapy. *Antib Ther* 2018;1:43–53.
- 40 Françoso A, Simioni PU. Immunotherapy for the treatment of colorectal tumors: focus on Approved and in-clinical-trial monoclonal antibodies. *Drug Des Devel Ther* 2017;11:177–84.
- 41 Wang J, Yan B, Liu S-M, *et al.* Transcriptomic and functional pathway analysis of human cervical carcinoma cancer cells response to microtubule inhibitor. *J Cancer* 2015;6:930–7.
- 42 Cao Y, Marks JD, Huang Q, *et al.* Single-Chain antibody-based immunotoxins targeting HER2/neu: design optimization and impact of affinity on antitumor efficacy and off-target toxicity. *Mol Cancer Ther* 2012;11:143–53.
- 43 Nakayama M, Harada N, Okumura K, *et al.* Characterization of murine TWEAK and its receptor (Fn14) by monoclonal antibodies. *Biochem Biophys Res Commun* 2003;306:819–25.
- 44 Cullen SP, Adrain C, Lüthi AU, *et al.* Human and murine granzyme B exhibit divergent substrate preferences. *J Cell Biol* 2007;176:435–44.