Development of Human Serine Protease-Based Therapeutics Targeting Fn14 and Identification of Fn14 as a New Target Overexpressed in TNBC

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Abstract
The cytokine TWEAK and its receptor, Fn14, have emerged as potentially valuable targets for cancer therapy. Granzyme B (GrB)-containing Fn14-targeted constructs were generated containing either the Fn14 ligand TWEAK (GrB-TWEAK) or an anti-Fn14 humanized single-chain antibody (GrB-Fc-IT4) as the targeting moieties. Both constructs showed high affinity and selective cytotoxicity against a panel of Fn14-expressing human tumor cells including triple-negative breast cancer (TNBC) lines. Cellular expression of the GrB inhibitor PI-9 in target cells had no impact on the cytotoxic effect of either construct. Cellular expression of MDR1 showed no cross-resistance to the fusion constructs. GrB-TWEAK and GrB-Fc-IT4 activated intracellular caspase cascades and cytochrome c-related proapoptotic pathways consistent with the known intracellular functions of GrB in target cells. Treatment of mice bearing established HT-29 xenografts with GrB-TWEAK showed significant tumor growth inhibition compared with vehicle alone (P < 0.05). Both GrB-TWEAK and GrB-Fc-IT4 displayed significant tumor growth inhibition when administered to mice bearing orthotopic MDA-MB-231 (TNBC) xenografts. The Cancer Genome Atlas analysis revealed that Fn14 mRNA expression was significantly higher in TNBC and in HER2-positive disease (P < 0.0001) compared with other TNBC molecular subtypes. IHC analysis of a 101 patient TNBC tumor microarray showed that 55 of 101 (54%) of tumors stained positive for Fn14, suggesting that this may be an excellent potential target for precision therapeutic approaches. Targeting Fn14 using fully human, GrB-containing fusion constructs may form the basis for a new class of novel, potent, and highly effective constructs for targeted therapeutic applications.

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Introduction
Precision protein therapeutics targeting cellular receptors are generally either antibody based or ligand based. The former class includes antibody drug conjugates (ADC) or immunotoxins (IT) using antigen recognition sites of immunoglobulin (Ig) molecules, whereas the latter includes fusion proteins containing the receptor-binding domains of native protein ligands guiding cytotoxic payloads to destroy specifically targeted cells. An example of a ligand-based targeted protein is the FDA-approved denileukin diftitox (Ontak), a fusion protein comprising the cytokine IL2 fused to diphtheria toxin (DT) for treatment against CD25-positive cutaneous T-cell lymphoma (1). This construct specifically targets the IL2 receptor (IL2R) upregulated on tumor cells and delivers modified DT to the cytoplasm resulting in apoptotic damage (1). However, the development of vascular leak syndrome caused by off-target binding to normal vasculature has been a troubling feature of numerous toxins such as DT and ricin A chain. In addition, the immunogenicity of DT as well as other bacterial and plant toxins (2) limits their potential value in long-term or repeated therapeutic applications.
regimens (3). Recently, Pastan and colleagues generated *Pseudomonas* exotoxin variants with reduced immunogenicity (4), which may alleviate part of the immunogenicity concern. Targeted cytotoxic fusion proteins composed entirely of human sequences represent an attractive alternative for application as anticancer agents.

The serine protease family members of granzymes along with perforin are well-known vital components of the cytotoxic lymphocyte and natural killer cell’s ability to induce apoptosis, contributing to rapid cell death of a target cell by direct and indirect activation of caspases and damage to mitochondria (5). Several laboratories, including ours, have utilized human Granzyme B (GrB) as an effective payload for the generation of recombinant cell death-inducing fusion proteins (6–8). Studies in our laboratories and by other groups have clearly demonstrated that GrB-containing fusion constructs have impressive and highly selective cytotoxic effects when delivered to the cytoplasm by either antibody or growth factor cell targeting carriers.

TWEAK (TNF-like weak inducer of apoptosis, TNFSF12), first described as an inducer of apoptosis in cancer cell lines, is a multifunctional cytokine involved in proinflammatory responses, angiogenesis, proliferation, migration, differentiation, and cell death (9, 10). TWEAK is synthesized as a type II transmembrane protein in the endoplasmic reticulum and is readily processed in most cell types by furin proteases resulting in the release of soluble TWEAK (11). The extracellular domain of human TWEAK is expressed as a homotrimERIC molecule and binds with high affinity to a receptor known as fibroblast growth factor-inducible 14 kDa protein (Fn14, TNFRSF12A; ref. 12).

Elevated Fn14 expression has been observed across numerous experimental settings, such as in inflammatory diseases, tissue remodeling (9), and in a variety of solid tumors (13) including tumor stroma and vascular tissue (14). In contrast, Fn14 expression in normal tissues is at relatively low levels. In cancer settings, overtexpression of Fn14 is associated with advanced disease and/or a worse clinical outcome in glioma (15), breast (16), esophageal (17), prostate (18), gastric (19), bladder (20), neuroblastoma (21), and urothelial (22) carcinomas. Recently, we demonstrated that Fn14 expression was elevated in 173 of 190 (92%) of primary melanoma specimens and 86 of 150 (58%) of melanoma metastases tested (23). Fn14 gene expression was shown to be elevated in breast tumor specimens when compared with normal breast tissue (24). Furthermore, when examining the expression of Fn14, the level of Fn14 mRNA and protein was higher in the cancer cell lines and most cancer tissues than in normal control tissues (25). The same study evaluated Fn14 expression in a breast cancer cohort and showed that Fn14 was expressed in 86.5% of the cases, and that positive Fn14 expression was associated with decreased overall survival (OS; ref. 25). Evaluation by breast cancer subtypes was not done. As a result of its limited expression in normal tissues, Fn14 has the potential to be an ideal candidate for the development of targeted therapy.

Triple-negative breast cancer (TNBC) is an aggressive subtype defined by the lack of expression of estrogen, progesterone, and HER2 receptors and accounts for 10% to 20% of invasive breast cancers. It is associated with a higher recurrence rate, particularly in the first 3 years after diagnosis and shorter survival outcomes than other subtypes of breast cancer (26, 27). TNBC is a heterogeneous disease and it lacks effective targeted therapies that can improve on benefits achieved with chemotherapy (27, 28). Thus, a better understanding of the molecular biology of this challenging subtype would assist in improving therapeutic outcomes with current agents and may provide an ability to generate precision therapeutics.

Both TWEAK and Fn14 have attracted considerable interest as therapeutic targets in inflammation, autoimmune diseases, and cancer (29). Although TWEAK or agonistic antibodies to Fn14 can induce tumor cell death *in vitro* and *in vivo* (13, 30), activation of Fn14 can also result in cell migration and survival (31). The upregulation of this pathway in cancer suggests a potential tumor-promoting function and also a rationale for inhibition as a therapeutic strategy. Recently, administration of a TWEAK-blocking antibody resulted in tumor growth inhibition in xenograft tumor models (32). On the basis of its expression pattern, we have proposed that the use of ADCs (33) or immunotoxins (23) to this receptor might be an appropriate therapeutic strategy.

Fn14 is the only known signaling-competent receptor for TWEAK (34). Therefore, we hypothesized that using the TWEAK receptor-binding domain fused to the cytotoxic GrB protein would effectively target Fn14-overexpressing solid tumors. Alternatively, antibodies binding the Fn14 extracellular domain may also be used for the same purpose.

In this study, we describe the development and characterization of two agents targeting the Fn14 receptor through either a ligand or an antibody-based approach: GrB-TWEAK, constructed via PCR using the human TWEAK receptor-binding domain as the targeting moiety; and GrB-Fc-IT4, constructed from a previously described engineered anti-Fn14 humanized single-chain antibody (23). Both fully human targetting agents utilize the proapoptotic serine protease GrB as the cytotoxic payload. We explored the mechanism of activity of GrB-TWEAK and GrB-Fc-IT4 and demonstrated the ability of these agents to kill Fn14-positive tumor cells *in vitro* (including TNBC) and against Fn14-positive colon cancer and TNBC tumor models *in vivo*. Finally, because our TNBC lines, all demonstrated high-level Fn14 expression, we examined TNBC arrays in The Cancer Genome Atlas (TCGA) and patient tumor array specimens for their expression of the Fn14 target at both the mRNA and protein levels to determine whether this represents a potential target in this aggressive breast tumor subtype.
Materials and Methods

Cell lines

Cell lines were obtained from the American Type Culture Collection and maintained in RPMI-1640 (MDA-MB-157, MDA-MB-436, MDA-MB-468), DMEM (Capan-1, Capan-2, L3.6P1, AsPC-1, MIA-PaCa-2, U-87 MG, and HEK-293T cells), DMEM/F12 (MDA-MB-231, Eb1, Calu-3, and RAW264.7 cells), RPMI-1640 (MDA-MB-435, MCF-7, BT-474, BxPc-3, NCI-N87, and Jurkat cells), McCoy’s 5A (T-24, HT-29, SKOV3, ME-180, and SKBR3 cells), F12 (PC-3), or Eagle’s Minimum Essential Medium (HT-1080). MDA-MB-231/Luc cells were kindly provided by Dr. Stuart Martin (University of Maryland School of Medicine, Baltimore, MD). All media contained 10% FBS. Cells were grown at 37°C with 5% CO2 at constant humidity. Media and supplements were purchased from Life Technologies, Inc.

Plasmid construction, protein expression, and purification

The GrB, GrB-TWEAK, and GrB-Fc-IT4 constructs were generated by an overlapping PCR method. Briefly, cDNA encoding human GrB was fused via a flexible (GGGGS)3 linker to a sequence encoding the human TWEAK extracellular domain (amino acids 97–249) to generate the GrB-TWEAK fusion gene. To generate the GrB-Fc-IT4 fusion gene, human GrB was fused to the N-terminus of the coding region of hinge CH2 and CH3 of a human IgG1 heavy chain followed by the humanized single-chain V H-V L variable fragment of the anti-Fn14 antibody (hscFvIT4; ref. 23). The mammalian expression vector pSecTag (Life Technologies) with a (His)6 tag and an enterokinase (EK) cleavage site was used to express pro-GrB, pro-GrB-TWEAK, and pro-GrB-Fc-IT4 (Fig. 1A) as previously described (35, 36). The Granzyme B activity assay

The enzymatic activity of the GrB component was determined in a continuous colorimetric assay using Ac-IEPD-pNA (N-acetyl-Ile-Glu-Pro-Asp-p-nitroanilide, Merck) as a specific substrate (37). Assays consisted of commercial human GrB (Enzyme Systems Products), GrB-TWEAK, or GrB-Fc-IT4 in Ac-IEPD-pNA at 25°C. The change in absorbance at 405 nm was measured on a Thermomax plate reader. Increases in sample absorbance were converted to enzymatic rates by using an extinction coefficient of 13,100 cm−1 M−1 at 405 nm. The specific activity of GrB-TWEAK and GrB-Fc-IT4 fusion proteins was calculated using native GrB as the standard.

Internalization analysis

Immunofluorescence-based internalization studies were also done on HT-29 and MDA-MB-231 as described previously (38). Briefly, internalization of GrB-TWEAK and GrB-Fc-IT4 into cells was examined by treating cells with either 50 or 100 nmol/L GrB, GrB-TWEAK, or GrB-Fc-IT4 for the indicated times. The cells were fixed, acid washed to remove surface-bound material, permeabilized, and immunostained for the presence of GrB. The cells were counterstained with propidium iodide to identify nuclei and visualized using a confocal microscope.

Flow-cytometric analysis for Fn14 cell surface expression

For the analysis of Fn14 cell surface expression, flow-cytometric analysis of cells stained with ITEM-4 was performed (39). Briefly, 5 × 105 cells were incubated for 1 hour on ice with ITEM-4 mAb or isotype control mouse IgG2a (1 μg/100 μL in 1% BSA in PBS). Cells were then washed twice with 0.5% BSA in PBS and incubated for an additional 30 minutes on ice with an FITC-conjugated goat anti-mouse IgG mAb. After two washes, cells were stained in 3.7% paraformaldehyde and analyzed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

In vitro cytotoxicity assays

Log-phase cells were seeded (~3 × 104/well) in 96-well plates and allowed to attach overnight. Cells were further incubated with various concentrations of GrB, GrB-TWEAK, GrB-Fc-IT4, or medium at 37°C for 72 hours. Cell viability was determined using the crystal violet staining method followed by solubilization of the dye in Sorenson’s buffer as described previously (33). The target index (the ratio of IC50 for GrB vs. the IC50 for GrB-TWEAK or GrB-Fc-IT4) was calculated for each cell line. This ratio represents the ability of the targeting moiety of GrB-based cytotoxic constructs to mediate delivery of the GrB component to the target cell cytoplasm and normalizes for any inherent cellular sensitivity to GrB alone.

Combination studies of GrB-TWEAK with conventional chemotherapeutic agents

For combination studies, GrB-TWEAK and chemotherapeutic agents were combined at various cytotoxic doses (e.g. IC25, IC50, IC75, etc.). HT-29 and MDA-MB-231 cells were pretreated with various chemotherapeutic agents for 6 hours, followed by the addition of GrB-TWEAK at a set dose. The cells were then incubated for a total of 72 hours (sequence I). Alternatively, cells were first treated with GrB-TWEAK for 6 hours, and then the various chemotherapeutic agents were added for 72 hours (sequence II). Finally, cells were coexposed to GrB-TWEAK and the chemotherapeutic agents at various doses, for 72 hours (sequence III). Cell viability was assessed by crystal violet staining. Normalized isobolograms were then generated using the CalcuSyn software, depicting combination index (CI) values of combination...
Drug studies. CI < 1, CI = 1, and CI > 1 indicate synergism, additive interaction, and antagonism, respectively. Chemotherapeutic agents include doxorubicin, GemZAR, cisplatin (CDDP), 5-fluorouracil (5-FU), TAXOL, and vinblastine.

Apoptosis assays
Western blot analysis was used to identify the activation of caspases-3 and -7 as well as PARP cleavage. In addition, apoptosis was analyzed using antibodies recognizing BID (Santa Cruz Biotechnology), and the Annexin V-FITC Kit (Molecular Probes, Inc.) was used to distinguish cells that were in early apoptosis (Annexin V+/PI−) or late apoptosis (Annexin V+/PI+). Apoptosis induction through mitochondrial membrane depolarization was also investigated using the cationic dye JC-1 (JC-1 Assay Kit; MitoProbe) according to the manufacturer’s instructions, as previously described (8). For in vivo detection of apoptosis, HT-29 fresh-frozen tumor sections were stained by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) using an in situ cell death detection kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

Figure 1. Preparation, purification, and enzymatic activity of GrB, GrB-TWEAK, and GrB-Fc-IT4 fusion proteins. A, schematic diagrams of the GrB, GrB-TWEAK, and GrB-Fc-IT4 constructs. The insert was cloned into the pSecTag vector and transiently expressed in HEK-293T cells. LS, the Igx leader sequence that promotes secretion of the fusion protein into the cell culture media; His6, polyhistidine tag to facilitate purification via IMAC; GrB, the human proapoptotic serine protease GrB; L, peptide linkers; ECD, the extracellular domain of human TWEAK (amino acids 97–249); H-CHx2-CH3, hinge CHx2 and CHx3 domains of a human IgG1 heavy chain; scFvIT4, humanized single-chain fragment variable anti-Fn14 antibody. Arrow, enterokinase (EK) cleavage site (EK is used to activate the GrB that is originally expressed in an inactive form). B, SDS-PAGE analysis of purified GrB and GrB-TWEAK on a 10% gel run under nonreducing conditions. C, SDS-PAGE and Western blot analysis (anti-GrB antibody) of purified GrB-Fc-IT4 on a 10% SDS-PAGE gel. D, enzymatic activity of GrB. Purified GrB-TWEAK and GrB-Fc-IT4 (0.05 μg, inactive or activated by recombinant enterokinase) was incubated with chromogenic substrate (0.2 mmol/L Ac-IEPD-pNA) at 37°C for the indicated time and absorption was measured at 405 nm.
Cytochrome c release assay and Bax translocation

Mitochondrial and cytosolic fractions were isolated from treated and untreated cells using Mitochondria/cytosol fractionation kit (BioVision) according to the manufacturer’s instructions. Briefly, after treatment with 100 nmol/L GrB-TWEAK, HT-29 or MDA-MB-231 cells were collected and resuspended with 0.5 mL of 1× cytosol extraction buffer mix (BioVision) and then homogenized in an ice-cold glass homogenizer. The homogenate was centrifuged, and the supernatant was collected and labeled as the cytosolic fraction. Aliquots of each cytosolic and mitochondrial fraction were analyzed by Western blotting with antibodies recognizing cytochrome c and Bax (Santa Cruz Biotechnology).

Western blot analysis

Western blot analyses were performed according to standard procedures. The primary antibodies used included anti-caspases-3 and -7, anti-PARP, anti-BID, anti-PI-9, anti-β-actin, (Santa Cruz Biotechnology), and anti-Fn14 (Cell Signaling Technology).

In vivo efficacy studies

All animal procedures were conducted according to a protocol approved by the AAALAC-approved Animal Care and Use Facility at MDACC (MD Anderson Cancer Center, Houston, TX). Female BALB/c nude mice (Harlan Sprague Dawley Inc.; 8 weeks old, five mice per group) were injected with 8 × 10⁶ HT-29 (left flank) or MDA-MB-231/Luc (mammary fat pad, 1:1 v/v with Matrigel) cells. Once tumor volumes reached 100 to 150 mm³, animals were treated (i.v. via tail vein, every other day, 5 or 6 total injections) with PBS, GrB (40 mg/kg), GrB-TWEAK (20 or 40 mg/kg), or GrB-Fc-IT4 (20 or 40 mg/kg). Animals were monitored and tumors were measured every 2 to 3 days. Data are presented as mean tumor volume (mm³) ± SEM. Survival was calculated using a predefined cutoff volume of 400 mm³ as a surrogate endpoint for mortality (39). Bioluminescence of MDA-MB-231/Luc tumors was quantitated in each region of interest using Living Image 4.3.1. software (PerkinElmer). Average percentage weight change was used as a surrogate endpoint for tolerability. Toxicity was defined as ≥20% of mice showing ≥20% body weight loss and/or mortality.

In vivo localization of GrB-TWEAK and GrB-Fc-IT4

For drug localization in HT-29 tumors, tumor tissue from the HT-29 efficacy study described above were harvested at the termination of the study and frozen immediately for sectioning. For all samples, histopathologic analysis included hematoxylin and eosin as well as immunofluorescence staining for human GrB and anti-CD31, performed as previously described (8). All images were taken under identical conditions. Slides were examined under fluorescence (Nikon Eclipse TS1000) and confocal (Zeiss LSM510) microscopes.

Cell line authentication

The following cell lines used in this study were authenticated by short tandem repeat DNA fingerprinting analysis by the Characterized Cell Line Core Facility at MDACC: SKOV-3, U87-MG, MDA-MB-231, SKBR3, N-87, Calu3, ME-180, A375, HT-29, BT-474. The Non-human cell line RAW264.7 cells were analyzed by G banding and confirmed to be of the stated origin.

Patients and IHC methods

We identified 101 patients with primary TNBC diagnosed between 1993 and 2009 whose tissues were available in the MDACC Breast Cancer Tumor bank. Patient and tumor data were collected by chart review. Tumors were considered triple receptor negative if nuclear staining was ≤5% for estrogen receptor, progesterone receptor, and HER2 receptor expression by IHC staining (membranous staining in less than 10% of cells), and/or negative for HER2 gene amplification as assessed by FISH. All patients were treated with a multidisciplinary approach. After definitive surgery, all patients received adjuvant chemotherapy with an anthracycline-based, a taxane-based, or an anthracycline/taxane-based and non-anthracycline/taxane-based regimens. The Institutional Review Board of The University of Texas, MDACC approved the laboratory retrospective study.

Paraffin tissue blocks from archived patient TNBC specimens were available from the MDACC Breast Cancer Tumor Bank and were used to construct tissue microarrays (TMA). These arrays consisted of duplicate 1-mm cores from each TNBC tumor block and an additional ten 1-mm cores from normal breast tissue as controls. IHC analysis for Fn14 protein expression was performed as previously described (15) using 2.5 μg/mL of the Fn14 mAB P4A8. Briefly, the slides were dewaxed, rehydrated, and antigen retrieved on-line on the BondMax autostainer (Leica Microsystems). Slides were subjected to heat-induced epitope retrieval using a proprietary citrate-based retrieval solution for 20 minutes and enzyme-induced epitope retrieval with Ficin for 5 minutes. Slides were incubated with the antibodies for 20 minutes and the antibody binding on the slides was visualized using the Bond Polymer Refine Red Detection Kit (Leica) using Fast Red chromogen as substrate. Immunolabeling was scored as 0 (<5%), 1 (5%–25%), 2 (25%–75%), and 3 (>75%). Stain intensity was defined as 0 (no staining), 1+ (low staining), 2+ (moderate staining), or 3+ (high staining). Slide evaluation and scoring were completed by a dedicated breast cancer pathologist.
Statistical analysis

TCGA breast cancer RNASeq data and clinical data were downloaded from TCGA data portal (March 2014; ref. 40). Breast cancer subtypes were determined by receptor (ER/PR/HER2) status from the clinical data. The downloaded RNASeqV2 raw counts were normalized using the TMM method (41) implemented in the Bioconductor package edgeR (42). Log$_2$-transformed RPKM (reads per kilobase per million mapped reads) was used as the final gene expression measurement.

The TNBC subtype prediction was performed using the online tool “TNBCtype” (http://cbc.mc.vanderbilt.edu/tncb/prediction.php; ref. 43). The RNASeqV2 data of TNBC samples were normalized separately without other subtypes as input to the online prediction tool as requested. Four TNBC samples did not pass the quality check of the TNBC subtype prediction tool and thus were excluded. ANOVA and Student $t$ test were used to test significance of differences between breast cancer subtypes and TNBC subtypes. Patients with TNBC were categorized according to IHC staining into one of two groups: Fn14-low (0 or 1+) and Fn14-high (2+ or 3+). Patient characteristics were tabulated and compared between groups using Fisher exact test. OS was measured from the date of surgery to the date of death or lost to follow-up. Relapse-free survival (RFS) was measured from the date of surgery to the date of first documented local or distant recurrence or lost to follow-up. The Kaplan–Meier product limit method was used to estimate the survival outcomes of all patients by groups; groups were compared using the log-rank statistic. $P$ values less than 0.05 were considered statistically significant; all tests were two sided.

Results

TWEAK is internalized following binding to Fn14-positive cells

Ligand-induced receptor internalization is an important process that regulates receptor-mediated functions of TNF-TNFR superfamily members (44) and critical for effective payload delivery. Thus, we first examined whether the TWEAK ligand could be internalized following binding to Fn14-positive cells. Internalization assessed by immunofluorescence microscopy showed uptake of FLAG-TWEAK by Fn14-positive U118 glioma cells within 30 minutes (Supplementary Fig. S1A). Western blot analysis indicated that Myc-tagged soluble TWEAK rapidly internalized within 5 minutes into Fn14-positive endothelial cells and remained intact at least 1 hour after treatment (Supplementary Fig. S1B). These data indicated that the TWEAK ligand could be exploited as a targeting and intracellular delivery moiety against Fn14-expressing cells.

Construction, expression, and purification of GrB, GrB-TWEAK, and GrB-Fc-IT4

Native human GrB and the GrB-based fusion constructs (Fig. 1A) were transiently expressed in human embryonic kidney cells (HEK-293T) and secreted into the culture media. Following purification by nickel affinity chromatography, the proteins were activated by removal of the poly-histidine tag by cleaving with recombinant enterokinase (15 U/mg, overnight). GrB and GrB-TWEAK migrated on SDS-PAGE under nonreducing conditions at the expected molecular weights of approximately 28 kDa and approximately 50 kDa, respectively (Fig. 1B). Similarly, SDS-PAGE analysis of GrB-Fc-IT4 under nonreducing conditions confirmed its purity and homodimeric molecular weight of approximately 160 kDa (Fig. 1C). Western blot analysis using a GrB- or an Fc-specific antibody for detection verified that the approximately 160-kDa band found for GrB-Fc-IT4 represents the complete construct (Fig. 1C).

GrB-TWEAK and GrB-Fc-IT4 proteins exhibit GrB enzymatic activity

The serine protease activity of the GrB component of the fusion proteins was compared with that of native GrB by incubating each protein with the chromogenic GrB substrate N-acetyl-Ile-Glu-Pro-Asp-p-nitroanilide (Ac-IEPD-pNA). Purified GrB-TWEAK and GrB-Fc-IT4 were found to hydrolyze the substrate at a rate that was comparable with that of the molar equivalent GrB standard ($\sim 1.2 \times 10^5$ U/µmol; Fig. 1D). Active GrB is generated in cytotoxic T cells by proteolytic removal of a two-residue propeptide resulting in exposure of the N-terminal isoleucine residue, which is necessary for enzymatic activity of GrB (45). Our previous studies have also shown that pro-GrB fusion constructs with purification tags at the GrB N-terminus render the molecule enzymatically inactive (8, 35, 46). Therefore, as expected, both pro-GrB-TWEAK and pro-GrB-Fc-IT4 demonstrated no enzymatic activity (Fig. 1D) before removal of the purification tag.

Both GrB-TWEAK and GrB-Fc-IT4 bind with high affinity to Fn14 and are specifically internalized into Fn14-expressing cells

We compared the binding of TWEAK and the fusion constructs to recombinant Fn14 extracellular domain by surface plasmon resonance (BIAcore) analysis. TWEAK and GrB-TWEAK bound to the Fn14 extracellular domain with similar equilibrium dissociation constants ($K_D$) of approximately 3 and 8 nmol/L by surface plasmon resonance (BIAcore) analysis, respectively (Supplementary Fig. S2A). GrB-Fc-IT4 bound to Fn14 (Supplementary Fig. S2B) with an equilibrium dissociation constant of 18 nmol/L, which is comparable with the Fn14 antibody ITEM-4 (23).
Figure 2. In vitro characterization of GrB-TWEAK and GrB-Fc-IT4 fusion proteins. GrB-TWEAK (A) and GrB-Fc-IT4 (B) specifically internalize into Fn14-expressing HT-29 cells. Cells were either left untreated or treated with 100 nmol/L GrB, GrB-TWEAK, or GrB-Fc-IT4 for the indicated times. The cells were fixed with PBS/4% paraformaldehyde, acid washed to remove surface-bound material, permeabilized, and immunostained for the presence of GrB (green). The cells were counterstained with propidium iodide (red) to identify nuclei and visualized using a confocal (Zeiss LSM 510) microscope. C, cytotoxicity of GrB-TWEAK when used as a single agent. Different concentrations of GrB-TWEAK were added to various tumor cell lines (AAB-527, HT-29, MDA-MB-435, MDA-MB-231) and Fn14-deficient mouse embryonic fibroblasts (MEF 3.5−/−) and cytotoxicity was measured as described in Supplementary Methods. (Continued on the following page.)
Thus, GrB-TWEAK in combination with various chemotherapeutic agents shows synergistic effects on HT-29 cells. We were unable to detect internalization of significant Fc-IT4 into the cytosol of Fn14-expressing HT-29 cells (Fig. 2A) and MDA-MB-231 cells (Supplementary Fig. S3). Immunofluorescence studies showed that exposure of Fn14-expressing cancer cells in the absence of perforin to internalize and deliver GrB to the cytoplasm of target cells is aided by the pore-forming protein perforin, either via formation of active perforin pores directly in the cell membrane, or disruption of vesicular membranes after co-endocytosis of GrB and perforin (47). We next examined the ability of GrB-TWEAK and GrB-Fc-IT4 to internalize and deliver GrB to the cytoplasm of Fn14-expressing cancer cells in the absence of perforin. Under physiologic conditions, delivery of GrB into the cytosol of target cells is aided by the pore-forming protein perforin, either via formation of active perforin pores directly in the cell membrane, or disruption of vesicular membranes after co-endocytosis of GrB and perforin (47). We next examined the ability of GrB-TWEAK and GrB-Fc-IT4 to internalize and deliver GrB to the cytoplasm of Fn14-expressing cancer cells in the absence of perforin. Immunofluorescence studies showed that exposure of cells to GrB-TWEAK resulted in efficient, rapid internalization of the GrB component to the cytoplasm of HT-29 (Fig. 2A) and MDA-MB-231 cells (Supplementary Fig. S3). We also found specific and rapid internalization of GrB-Fc-IT4 into the cytosol of Fn14-expressing HT-29 cells (Fig. 2B). We were unable to detect internalization of significant amounts of GrB when cells were exposed to native GrB alone (Fig. 2A and B).

**GrB-TWEAK and GrB-Fc-IT4 are highly cytotoxic to Fn14-expressing cancer cells**

We compared the cytotoxic effects of native GrB, GrB-TWEAK, and GrB-Fc-IT4 on cell lines expressing various levels of Fn14 and correlated the cytotoxic effects of the fusion proteins with Fn14 expression levels. A head-to-head comparison of the two GrB-based Fn14-targeted constructs against a number of cancer cell lines is shown in Table 1. Comparison of the IC₅₀ values showed that both GrB-TWEAK and GrB-Fc-IT4 exhibited specific cytotoxicity to Fn14-positive cells, compared with GrB treatment alone. Cell lines that were negative for Fn14

### Table 1. Comparative IC₅₀ values of the GrB-TWEAK and GrB-Fc-IT4 fusion constructs against various cancer cell lines

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<th>GrB-Fc-IT4</th>
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Under physiologic conditions, delivery of GrB into the cytosol of target cells is aided by the pore-forming protein perforin, either via formation of active perforin pores directly in the cell membrane, or disruption of vesicular membranes after co-endocytosis of GrB and perforin (47). We next examined the ability of GrB-TWEAK and GrB-Fc-IT4 to internalize and deliver GrB to the cytoplasm of Fn14-expressing cancer cells in the absence of perforin. Immunofluorescence studies showed that exposure of cells to GrB-TWEAK resulted in efficient, rapid internalization of the GrB component to the cytoplasm of HT-29 (Fig. 2A) and MDA-MB-231 cells (Supplementary Fig. S3). We also found specific and rapid internalization of GrB-Fc-IT4 into the cytosol of Fn14-expressing HT-29 cells (Fig. 2B). We were unable to detect internalization of significant amounts of GrB when cells were exposed to native GrB alone (Fig. 2A and B).

**GrB-TWEAK and GrB-Fc-IT4 are highly cytotoxic to Fn14-expressing cancer cells**

We compared the cytotoxic effects of native GrB, GrB-TWEAK, and GrB-Fc-IT4 on cell lines expressing various levels of Fn14 and correlated the cytotoxic effects of the fusion proteins with Fn14 expression levels. A head-to-head comparison of the two GrB-based Fn14-targeted constructs against a number of cancer cell lines is shown in Table 1. Comparison of the IC₅₀ values showed that both GrB-TWEAK and GrB-Fc-IT4 exhibited specific cytotoxicity to Fn14-positive cells, compared with GrB treatment alone. Cell lines that were negative for Fn14

![Table 1. Comparative IC₅₀ values of the GrB-TWEAK and GrB-Fc-IT4 fusion constructs against various cancer cell lines](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor type</th>
<th>Fn14</th>
<th>GrB</th>
<th>GrB-TWEAK</th>
<th>GrB-Fc-IT4</th>
<th>Targeting index²</th>
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<tr>
<td>A549</td>
<td>Lung</td>
<td>++++</td>
<td>1,406</td>
<td>97.2</td>
<td>114</td>
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<td>H1975</td>
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<td>&gt;3,200</td>
<td>15.9</td>
<td>13.5</td>
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<tr>
<td>HCC827</td>
<td>Lung</td>
<td>++++</td>
<td>1,046</td>
<td>97.2</td>
<td>114</td>
<td>14</td>
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<tr>
<td>HCC2279</td>
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<td>++++</td>
<td>&gt;3,200</td>
<td>15.9</td>
<td>13.5</td>
<td>&gt;201</td>
</tr>
<tr>
<td>MDA-MB-231/Luc</td>
<td>Breast</td>
<td>++++</td>
<td>&gt;3,200</td>
<td>15.9</td>
<td>3.4</td>
<td>224</td>
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<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>++++</td>
<td>&gt;3,200</td>
<td>15.9</td>
<td>3.4</td>
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<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>++</td>
<td>3,544</td>
<td>15.9</td>
<td>3.4</td>
<td>224</td>
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<tr>
<td>MEF 3.5</td>
<td>Mouse embryonic fibroblast</td>
<td>--</td>
<td>3,200 &gt;900</td>
<td>&gt;900</td>
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Under physiologic conditions, delivery of GrB into the cytosol of target cells is aided by the pore-forming protein perforin, either via formation of active perforin pores directly in the cell membrane, or disruption of vesicular membranes after co-endocytosis of GrB and perforin (47). We next examined the ability of GrB-TWEAK and GrB-Fc-IT4 to internalize and deliver GrB to the cytoplasm of Fn14-expressing cancer cells in the absence of perforin. We compared the cytotoxic effects of native GrB, GrB-TWEAK, and GrB-Fc-IT4 on cell lines expressing various levels of Fn14 and correlated the cytotoxic effects of the fusion proteins with Fn14 expression levels. A head-to-head comparison of the two GrB-based Fn14-targeted constructs against a number of cancer cell lines is shown in Table 1. Comparison of the IC₅₀ values showed that both GrB-TWEAK and GrB-Fc-IT4 exhibited specific cytotoxicity to Fn14-positive cells, compared with GrB treatment alone. Cell lines that were negative for Fn14...
expression (such as MEF3.5−/− and Jurkat; Table 1 and Supplementary Table S1) were not specifically targeted by the fusion proteins. As shown in Table 1 and Supplementary Table S1, melanoma was generally found to be the most sensitive cancer type. The ovarian cancer cell line SKOV3 was found to be the most sensitive to GrB-TWEAK (targeting index ~ 350,000; Supplementary Table S1). We also observed excellent cytotoxic activity of GrB-Fc-IT4 against the four TNBC lines tested (MDA-MB-231, 157, 436, and 468; Supplementary Table S2). Of note, there was no correlation between sensitivity to cancer cell killing (as assessed by IC50 values) and the relative levels of Fn14 expression as determined by flow cytometry (Table 1 and Supplementary Tables S1 and S2). A representative cytotoxicity profile of GrB-TWEAK following addition to various cells in culture for 72 hours is shown in Fig. 2C. To quantitatively compare the cytotoxicity of GrB-TWEAK and GrB-Fc-IT4, dose–response growth inhibition curves were established on MDA-MB-231/Luc cells (Fig. 2D). IC50 values of 15.9 and 3.4 nmol/L were obtained for GrB-TWEAK and GrB-Fc-IT4, respectively.

We next evaluated the combination of GrB-TWEAK with various chemotherapeutic agents on Fn14-expressing various cells. We treated cells with known cytotoxic doses of GrB-TWEAK alone or with various concentration ranges of each chemotherapeutic agent. Multiple drug effect analyses were conducted to determine the nature of the interaction occurring in the combination treatment, and CI values were calculated (48). We found no difference in overall activity of combinations of chemotherapeutic agents with GrB-TWEAK based on the order of treatment (data not shown). GrB-TWEAK in combination with doxorubicin, 5-FU, cisplatin, GemZAR, taxol, or vinblastine revealed the mean CI to be less than 1.0 at multiple doses on HT-29 cells, indicating synergistic effects of the combinations in tumor cell growth inhibition (Fig. 2E). A synergistic cytotoxic effect of GrB-TWEAK was also observed with cisplatin, GemZAR, and 5-FU on the TNBC cell line MDA-MB-231 (Supplementary Fig. S4). Intriguingly, doxorubicin in combination with GrB-TWEAK resulted in an antagonistic cytotoxic effect on MDA-MB-231 cells (Supplementary Fig. S4). These results suggest that, in some cases, targeting Fn14-expressing tumor cells with GrB-TWEAK in combination with a variety of standard of chemotherapy agents may result in reduced drug resistance and enhanced efficacy.

**Mechanistic studies of GrB-based fusion protein cytotoxicity**

Apoptotic cell death is a hallmark of GrB-induced cytotoxicity (5). Compared with nontreated cells, GrB-TWEAK induced apoptosis in both HT-29 and MDA-MB-231 cells in a dose- (Fig. 3A) and time-dependent (Fig. 3B) manner, whereas no significant increase in Annexin V+ cells was observed when cells were treated with GrB or TWEAK alone (Fig. 3B). Western blot analysis following exposure of these cell lines to GrB-TWEAK (Fig. 3C) or GrB-Fc-IT4 (Fig. 3D) in a dose-dependent manner revealed cleavage of caspase-3, -7, and the apoptotic substrate PARP, suggesting that the cytotoxic effect of these fusion proteins was mediated, at least in part, by apoptosis via the caspase-dependent pathway. However, although caspase activation represents a defining characteristic of GrB-mediated cell death, GrB-TWEAK-mediated PARP cleavage was not completely blocked by addition of the pan-caspase inhibitor z-VAD-Imk (Supplementary Fig. S5), indicating that caspase activation is not solely responsible for the cytotoxic effect against target cells.

Mitochondrial dysfunction and the involvement of Bid, Bax, and/or Bak have also been implicated in GrB-mediated apoptosis (49). Therefore, we investigated the roles of these effectors in GrB-based construct-mediated cytotoxicity. As shown in Fig. 3C and D, Bid was cleaved in target cells treated with GrB-TWEAK and GrB-Fc-IT4. Cleaved Bid is believed to promote apoptosis via mitochondrial outer membrane permeabilization (MOMP; ref. 50). To examine the effect of GrB-TWEAK on the mitochondria of target cells, we examined mitochondrial depolarization (the loss of mitochondrial transmembrane potential as measured by green fluorescence of the JC-1 cationic dye in its monomeric form) and MOMP (via cytochrome c release) in treated cells. GrB-TWEAK treatment associated with mitochondrial membrane depolarization of HT-29 cells in a dose-dependent manner (Fig. 3E). Treatment at the approximate IC50 dose (50 nmol/L) resulted in 36.4% of cells undergoing mitochondrial depolarization, compared with 6.7% of untreated and 7.7% of GrB-treated cells. GrB-TWEAK treatment also resulted in the release of cytochrome c into the cytoplasm and translocation of Bax from the cytoplasm to the mitochondria (Fig. 3F), consistent with induction of apoptosis. Interestingly, GrB-TWEAK treatment of MDA-MB-231 cells did not induce mitochondrial depolarization (Fig. 3E), but did...
result in the release cytochrome c from mitochondria (Fig. 3F). These latter data are consistent with other observations that GrB processes Bid to release cytochrome c from isolated mitochondria but does not directly cause mitochondrial swelling or depolarization (51). Collectively, these data suggest that GrB-based constructs activated caspase cascades and cytochrome c-related proapoptotic mechanisms consistent with the known intracellular functions of GrB in target cells.

**Cells expressing MDR are not cross-resistant to GrB-TWEAK or GrB-Fc-IT4**

Resistance mechanisms related to the upregulation of cellular efflux pumps (MDR/MRP) resulting in decreased intracellular drug levels have been shown to be a central problem in reducing patient response to therapy for a number of chemotherapeutic agents (52). The P-gp-overexpressing human melanoma MDA-MB-435/LCC6MDR1 cells and the paclitaxel-derived ovarian cancer HeyA8-MDR cells have previously been shown to be less sensitive to paclitaxel and doxorubicin as a result of MDR expression (38). To evaluate the effect of MDR1 expression on GrB-TWEAK and GrB-Fc-IT4-induced cell killing, we compared the sensitivities of MDA-MB-435/LCC6MDR1 and HeyA8-MDR cells and their parental counterparts (MDA-MB-435 and HeyA8) to GrB-TWEAK and GrB-Fc-IT4. As shown in Supplementary Table S3, the IC50s of GrB-TWEAK and GrB-Fc-IT4 on the MDR cells were similar to their parental cells (less than ~ 1-fold for both MDR cell lines, respectively), suggesting that the cytotoxic activity of these GrB-based proteins may be effective in circumventing MDR1-mediated multidrug resistance in cancer.

**The GrB inhibitor proteinase inhibitor 9 (PI-9) has no impact on the cytotoxic effects of GrB-TWEAK and GrB-Fc-IT4**

The proteinase inhibitor 9 (PI-9, serpin B9) is a known natural inhibitor of GrB and expression of high levels of PI-9 has been shown to block perforin/granzyme-mediated cytotoxicity in immune effector functions (53). We examined PI-9 expression levels in different cancer cell lines by Western blot analysis (Supplementary Fig. S6) to evaluate its potential impact on GrB-based therapeutics. However, we did not observe an association between the response of cells to the cytotoxicity of the GrB constructs (Table 1) and the endogenous expression of PI-9.

**GrB-TWEAK localizes in HT-29 xenograft tumors after intravenous administration and inhibits tumor growth in vivo**

We next asked whether the internalization and cytotoxicity of these constructs observed in vitro could be translated in an in vivo setting. We first assessed the localization of GrB-TWEAK and GrB in established HT-29 human colon adenocarcinoma xenografts and the impact on tumor growth. Tumor-bearing mice were treated every other day from day 3 to day 11. HT-29 tumors were harvested upon termination of the efficacy study. Tumor sections from the GrB-TWEAK-injected mice showed staining with an anti-GrB antibody, whereas no apparent staining was observed in the sections from saline groups (Fig. 4A). A relatively low level of GrB was detectable in cells after exposure to native GrB. Overall, the GrB payload was selectively delivered to tumor cells by the TWEAK ligand. A significant suppression of tumor volume was observed at the 20 or 40 mg/kg GrB-TWEAK doses compared with saline controls ($P < 0.005$, from day 5, Fig. 4B). We observed a modest (but not statistically significant) reduction in tumor volume after treatment with 40 mg/kg GrB alone, compared with controls ($P > 0.05$). Toxicity was monitored by frequent body weight measurements in groups of mice treated with GrB-TWEAK. No differences in changes to body weight were observed in control versus treated mice for the duration of the study (Supplementary Fig. S7A). At the end of the study, tumors were harvested for histopathological analysis. As shown in Fig. 4C, there was a dramatic increase in the number of apoptotic cells (as assessed by TUNEL staining) in tumors from mice treated with 20 or 40 mg/kg GrB-TWEAK compared with tumors from mice treated with saline or GrB.

**Comparative in vivo efficacy of GrB-Fc-IT4 and GrB-TWEAK against TNBC xenografts**

To assess the efficiency of drug localization in MDA-MB-231/Luc tumors, immunofluorescence staining was performed on tumor tissues harvested 24 hours after intravenous drug administration. Both GrB-TWEAK and GrB-Fc-IT4 localized specifically in tumor tissue while no GrB staining was observed in tumors after administration of saline (data not shown) or native GrB (Fig. 5A). We next compared the effects of GrB-TWEAK with GrB-Fc-IT4 on the growth of established tumors in a MDA-MB-231/Luc orthotopic xenograft model of TNBC. Primary tumor sizes were assessed by caliper measurement or bioluminescence imaging. Significant tumor growth inhibition was observed at both doses of GrB-Fc-IT4 (20 or 40 mg/kg) compared with saline control ($P < 0.05$; Fig. 5B). Tumor growth was considerably suppressed for the entire study period of more than 50 days in the 40 mg/kg GrB-Fc-IT4 treatment group ($P = 0.007$; Fig. 5B). Mice treated with 40 mg/kg GrB-TWEAK (equivalent to three times the molar dose of 40 mg/kg GrB-Fc-IT4) also had significant inhibition of tumor growth relative to the saline control ($P = 0.03$ on day 50). Survival in the mice treated with GrB-TWEAK or GrB-Fc-IT4 was significantly longer than in those treated with saline or with GrB ($P < 0.009$; Fig. 5C). Quantitation of the day 47 bioluminescence images (Fig. 5D) demonstrated no significant impact of GrB-TWEAK despite an observed delay in tumor growth. In contrast, bioluminescence was reduced to 27% of saline control in GrB-Fc-IT4 20 mg/kg-treated tumors and 6% of saline control in GrB-Fc-IT4 40 mg/kg-treated tumors ($P < 0.06$; Fig. 5D). Because both the ITEM-4 antibody and human TWEAK can recognize murine Fn14 (54), we
assessed the tolerance of mice to these GrB-based constructs by monitoring change in body weight. The difference in mouse body weight changes in control versus treated mice was approximately 10% over the duration of the experiment (Supplementary Fig. S7B). Overall, the data indicated that although treatment with GrB-Fc-IT4 displayed a more pronounced and prolonged tumor growth inhibition, longer survival was observed with GrB-TWEAK treatment.

Interrogation of TCGA portal for Fn14 mRNA expression levels in breast cancers

Because all TNBC cell lines examined demonstrated a high level expression of Fn14 and excellent sensitivity to the constructs, we interrogated TCGA for Fn14 expression in breast cancer. Clinical information and RNASeq data in the TCGA data portal were available for 636 breast cancers. Tumors were classified as ER+/HER2- (n = 389), ER+/HER2+ (n = 110), ER-/HER2+ (n = 37), and TNBC.
Figure 5. In vivo activity of GrB-TWEAK and GrB-Fc-IT4 in an MDA-MB-231/Luc breast tumor orthotopic xenograft model. A, GrB, GrB-TWEAK, or GrB-Fc-IT4 was administered (i.v.) to MDA-MB-231/Luc tumor-bearing mice. One day later, animals were sacrificed, and tumor tissues were removed, fixed, and stained with immunofluorescent reagents to detect nuclei (Hoechst, blue) and GrB (green), and murine blood vessels (CD31, red). Colocalization of GrB into CD31⁺ tumor vessels appears yellow. B, MDA-MB-231/Luc cells were implanted under the mammary fat pad and groups of mice (n = 5) were treated (i.v. via tail vein) with saline, GrB-TWEAK (40 mg/kg), and GrB-Fc-IT4 (20 and 40 mg/kg) every other day starting when the tumors were approximately 100 mm³. Tumor size was assessed by direct caliper measurement. Efficacy data are plotted as mean tumor volume (in mm³) ± SEM, and arrows indicate dosing days. C, survival data are plotted as percentage of animals surviving in each group. D, bioluminescence quantitation of tumors, Day 47. **, P < 0.06, compared with saline control.
Figure 6 shows the gene expression of Fn14 according to breast cancer clinical subtype. There was a significant difference in Fn14 mRNA expression among breast cancer subtypes (ANOVA test $P < 0.0001$). There was also a significant difference in Fn14 mRNA expression between all pairwise of subtypes ($t$ test $P < 0.0001$ for all comparisons). Fn14 mRNA expression was analyzed for 96 TNBC specimens for which clinical information and RNASeq data in the TCGA data portal were available. Tumors were classified as BL1, basal-like 1 ($n = 16$); BL2, basal-like 2 ($n = 10$); IM, immunomodulatory ($n = 18$); LAR, luminal androgen receptor ($n = 7$); M, mesenchymal ($n = 21$); MSL, mesenchymal stem-like ($n = 6$); UNS, unclassified ($n = 18$). There was a significant difference in Fn14 mRNA expression among TNBC subtypes (ANOVA test $P = 0.01$; Fig. 6B). The mRNA expression of Fn14 was found to be significantly higher in the BL2 tumors when compared with other TNBC subtypes ($P < 0.02$ for all comparisons).

Expression of Fn14 assessed by IHC analysis of TNBC patient tumor microarray

As proteins are the effectors of biology, we stained a TNBC array for Fn14 protein expression. Figure 7 shows examples of tumors with different levels of Fn14 staining. We found that 46 of 101 tumors (46%) demonstrated low Fn14 staining (0 to 1+), whereas 55 of 101 tumors (54%) showed high levels of Fn14 (2+ to 3+). Patient characteristics stratified by Fn14 groups are summarized in Supplementary Table S4. At a median follow-up of 76.2 months (range, 12–341 months), 20 patients (19.8%) had experienced a recurrence, and 20 patients (19.8%) had died. The 5-year OS estimates were 91% and 84% in patient tumors with low Fn14 and high Fn14 protein expression, respectively ($P = 0.09$). The 5-year RFS was 87% and 88% in the high and low Fn14 groups ($P = 0.84$).

Discussion

There have been limited studies examining Fn14 expression in breast cancer. Willis and colleagues examined public databases to analyze TWEAK and Fn14 gene expression and found that high levels of Fn14 correlated with HER2-positive/ER-negative breast cancer. Further IHC studies confirmed the association and, in addition, showed the absence of Fn14 in most noninvasive breast cancers and normal breast tissues (55). In a more recent study, investigators measured Fn14 protein expression by IHC. They reported varied expression in 86.5% of the cases, and correlated the expression with ER negativity and HER2 positivity. Interestingly, more patients with node-negative disease were found to harbor Fn14-positive tumors, but patients with Fn14-positive tumors had a worse cumulative survival (25). Compared with our study, the frequency of Fn14 overexpression in both previous reports is higher; however, these reports did not evaluate these cases by breast cancer subtypes. Furthermore, the use of TMA can limit the evaluation of protein expression levels due to tumor heterogeneity, the use of different anti-Fn14 antibodies for IHC staining, or differences in the immunoreactivity scorings used to define the groups. Although prior studies have reported an association between Fn14 expression and inferior clinical outcomes in patients with hepatocellular carcinoma, breast, gastric, and prostate cancers (18, 19, 25, 56), no study has investigated the correlation between Fn14 expression with patient characteristic and clinical outcomes in TNBC. When looking at the 5-year RFS and OS in our TNBC cohort, it is noticeable that their outcome is quite good as compared with other TNBC populations. This can be explained because of the fact that most of our patients (more than 95%) presented with early-stage disease (pathological stage I and II), with more than 65%
of them having negative lymph nodes at the time of surgery (Supplementary Table S4). Although our study showed that Fn14 levels were not independently associated with outcomes (P = 0.30 for OS and 0.83 for RFS), these results could be limited by the small sample size. Larger scale studies are ongoing to examine this potential association.

We previously demonstrated that fusion constructs composed of the ribosome inactivating plant-derived protein gelonin (rGel) toxin and a humanized, dimeric single-chain antibody targeting Fn14 (designated hSGZ) resulted in potent antitumor activity against a number of cell lines and tumor xenografts (23, 33, 38). Concerns over the potential immunogenicity of rGel led us to develop a number of human cytotoxic proteins as payloads for use in targeted therapeutic applications (8, 57). Completely human constructs containing GrB produced in human cell lines should alleviate any concerns about immunogenicity and studies in animal models demonstrate a lack of toxicity. In the current study, we developed and characterized a ligand fusion construct (GrB-TWEAK) and an antibody fusion construct (GrB-Fc-IT4), both targeting the Fn14 receptor, and explored their cytotoxic and mechanistic effects against a wide panel of cells expressing Fn14. Both GrB-TWEAK and GrB-Fc-IT4 demonstrated impressive and selective cytotoxicity against Fn14-expressing human tumor cell lines, showed no cross-resistance against cells expressing the multidrug resistance protein MDR1, interacted synergistically with several chemotherapeutic agents, internalized into Fn-14+ cells in vitro and in vivo, and inhibited tumor growth in vivo.

We have previously determined Fn14 receptor expression in various tumor cell lines by flow cytometry, including HT-29 and MDA-MB-231 cell lines (33), both of which expressed moderately high levels of Fn14 on the cell surface. In addition, HT-29 has been used as a model to study Fn14–TWEAK interaction (58, 59), while MDA-MB-231 cells are a well-characterized TNBC cell line. The HT-29 xenograft tumor model revealed a moderate antitumor effect of the GrB-TWEAK construct (Fig. 4) that was statistically significant, but did not appear to be robust. This may be due to rapid clearance from circulation, a known limitation of in vivo applications of recombinant soluble TNF ligands (60). Furthermore, natural ligands of a given receptor target are typically present in the body, which may compete for access and binding of a drug to that target (61). Combination therapy with GrB-TWEAK may improve efficacy in a number of tumor models. Studies in vitro with GrB-TWEAK and several standard chemotherapeutic agents showed a strong synergistic effect (Fig. 2E) suggesting that trials to investigate the combination of GrB-TWEAK with chemotherapeutic agents are appropriate to pursue. Interestingly, we did not observe a difference in sensitivity to GrB-TWEAK based on the order of treatment with chemotherapeutic agents. This is in contrast with our previous study with

**Figure 7.** Fn14 IHC expression: A, negative 0+; B, negative 1+; C, positive 2+; D, positive 3+.
another GrB-based therapeutic targeting melanoma cells (62) and underscores the potential for differences against different tumor types and with different molecular targets.

Bivalent targeting of Fn14 has shown improved cytotoxicity of a single chain immunotoxin containing RGeL (23). The GrB-Fc-IT4 construct expands on this bivalent targeting concept because the GrB component is linked to the IgG heavy chain hinge region and scFvIT4 is fused to the carboxy terminus of the Fc domain of the IgG (Supplementary Fig. S8). By fusing scFvIT4 to the C-terminus of the Fc domain, we created a new bivalent Fn14-targeted GrB containing molecule by virtue of the efficient homodimerization of the two Fc domains. This approach retains several desirable features of antibodies, notably an increased apparent affinity through the avidity conferred by the dimerization of the two Fc domains. In contrast with the GrB-TWEAK construct, the IgG-like nature of the GrB-Fc-IT4 construct should promote a relatively long plasma residency time (63). Pharmacokinetic studies comparing the two constructs are currently underway.

The mechanism of cell death due to GrB-TWEAK or GrB-Fc-IT4 treatment is primarily apoptotic, as assessed by both caspase activation and cytochrome c release from mitochondria. This is consistent with the known intracellular functions of GrB in target cells. The release of cytochrome c without mitochondrial depolarization in MDA-MB-231 cells following GrB-TWEAK treatment could be the consequence of mitochondrial swelling that may result in the release of intramitochondrial proteins like cytochrome c without mitochondrial depolarization (64). On the other hand, cytochrome c release in the absence of mitochondrial swelling has also been reported (65). This finding seems to result from a unique interaction of GrB-TWEAK with MDA-MB-231 cells because GrB-TWEAK induced mitochondrial depolarization in HT-29 cells, and MDA-MB-231 cells have previously been shown to undergo mitochondrial depolarization when treated with a proapoptotic agent (66).

The effectiveness of GrB can be hampered by the cytosolic expression of a natural inhibitor, PI-9 (serpin B9). Therefore, expression of endogenous PI-9 levels in cancer cells could inhibit the GrB activity of our targeted constructs and could serve as a potential resistance mechanism. However, we were unable to demonstrate a relationship between PI-9 levels and cell sensitivity to both GrB-TWEAK and GrB-Fc-IT4 in Fn14-positive cells. We speculate that receptor-mediated internalization of these molecules via endosomal pathways may circumvent the protective role of PI-9 in target cells.

For a number of chemotherapeutic agents, resistance mechanisms related to upregulation of cellular efflux pumps (MDR/MRP) resulting in decreased intracellular drug levels have been shown to be a central problem resulting in reduced response (52). In addition, expression of MDR/MRP has been demonstrated to reduce response to ADCs (67). The current studies show that expression of MDR does not seem to result in cross-resistance to GrB-based fusion constructs. This property would be a significant advantage over some conventional therapeutic agents and over some ADCs.

The Fn14 agonistic antibodies, BIIB036 and PDL192, have demonstrated impressive \textit{in vivo} efficacy that may be partially mediated by ADCC and activation of NF-kB signaling even though neither antibody showed significant cytotoxicity \textit{in vitro} against the same cell lines (13, 68). This suggests that direct cell killing is unlikely to be the predominant mechanism of action (69). The fact that GrB-Fc-IT4 does not show a cytotoxic advantage over GrB-TWEAK \textit{in vitro} but was the more potent therapeutic against MDA-MB-231 orthotopic human breast xenografts suggests that Fc domain-mediated effects of GrB-Fc-IT4 cannot be completely ruled out. Although the enhanced tumor inhibition activity of GrB-Fc-IT4 \textit{in vivo} may be partially attributed to the Fc domain, differences in pharmacokinetic properties of the molecules should be carefully examined.

Data from our analysis of TNBC cell lines (Supplementary Table S2) demonstrated that Fn14 was highly expressed in all four lines which were sensitive to the GrB constructs and this provided an impetus to further investigate the overall expression pattern of Fn14 in TNBC. Fn14 was found to be highly expressed on more than 50% of TNBC tumors. Although overexpression does not seem to have direct biologic consequences to OS in our limited sample size, it does identify a new cell-surface target for precision therapeutic intervention providing an expanded spectrum for the application of Fn14-targeted therapeutic agents, which now includes a high percentage of patients with TNBC. In addition, these data demonstrate that completely human fusion constructs targeting the Fn14 receptor and containing the cytotoxic GrB payload have excellent \textit{in vitro} and \textit{in vivo} targeted cytotoxic effects. Finally, these studies indicate that scFv-targeted, Fc-containing fusions with GrB may have a design advantage with respect to efficacy of ligand-based fusion constructs and may form the basis for a new generation of novel, highly effective, and nontoxic constructs for targeted therapeutic applications.

Disclosure of Potential Conflicts of Interest
L.H. Cheung has ownership interest in a pending patent. No potential conflicts of interest were disclosed by the other authors.

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References

16. Shimada K, Fujii T, Tsujikawa K, Anai S, Fujimoto K, Konishi N. ALKBH3 contributes to survival and angiogenesis of human urothelial carcinoma cells through NADPH oxidase and t treadmill of mtc.aacrjournals.org Downloaded from mtc.aacrjournals.org on February 4, 2021. © 2014 American Association for Cancer Research.
Molecular Cancer Therapeutics

Development of Human Serine Protease-Based Therapeutics Targeting Fn14 and Identification of Fn14 as a New Target Overexpressed in TNBC

Hong Zhou, Khalid A. Mohamedali, Ana Maria Gonzalez-Angulo, et al.


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