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PRINCIPAL INVESTIGATOR: Jeffrey A. Winkles, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland, Baltimore
Baltimore, MD 21201

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Jeffrey A. Winkles, Ph.D.

E-Mail: jwinkles@som.umaryland.edu

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14. ABSTRACT

Our laboratory research is focused on the potential roles of the TNF-related cytokine named TWEAK and its specific cell surface receptor named Fn14 in tumor biology. We reported previously that the Fn14 gene is expressed at low levels in normal lung tissue but highly expressed in many non-small cell lung cancers (NSCLCs). Additionally, in collaboration with Dr. Rosenblum's research group at MD Anderson Cancer Center we have successfully developed several Fn14-targeted fusion proteins that exhibit cytotoxic activity on cancer cells *in vitro* and *in vivo*. In this Lung Cancer Idea Award application we proposed to test the effects of these fusion proteins on NSCLC cell viability. During the current funding period we have been able to demonstrate that two different Fn14 targeted proteins that use granzyme B (GrB) as the cell killing agent (TWEAK-GrB, GrB-Fc-IT4) exhibit pro-apoptotic activity when added to numerous Fn14-positive NSCLC cell lines.

15. SUBJECT TERMS
TWEAK, Fn14, targeted therapy, granzyme B, lung cancer

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Table of Contents

Page

1. Introduction: Lung cancer kills ~158,000 individuals per year in the USA and thus it is critical that we identify new molecular targets and therapeutic agents (1). Our laboratory research is focused on the potential roles of a TNF-related cytokine named TWEAK and its specific cell surface receptor named Fn14 in tumor biology (the TWEAK-Fn14 axis is reviewed in references 2 and 3). We reported in 2012 that Fn14 is frequently highly expressed in human non-small cell lung cancer (NSCLC) tumors (4). More recently, high Fn14 levels have also been found in the metastatic lesions of NSCLC patients (5). In this Lung Cancer Idea Award application we proposed to investigate if Fn14-targeted fusion proteins using the pro-apoptotic serine protease granzyme B (GrB) as the cell-killing moiety may be potential therapeutic agents for NSCLC patients with Fn14-positive tumors. If so, this could be a new therapeutic approach that could potentially advance to human clinical trials.

2. Keywords: TWEAK, Fn14, targeted therapy, granzyme B, lung cancer

3. Accomplishments:

a. What were the major goals of the project and what was accomplished?

Specific Aim 1: To determine if GrB-TWEAK has cytotoxic activity on Fn14-positive NSCLC cells representing various NSCLC molecular subtypes and EGFR TKI sensitivities.

MILESTONES

- Complete initial cytotoxicity assays (Months 1-3) - completed
- Complete cellular uptake assays (Months 4-6) - completed
- Complete mechanistic basis assays (Months 7-12) - completed

In the funded application we proposed to focus on the GrB-TWEAK fusion protein but mentioned that there was another Fn14-targeted protein under development named GrB-Fc-IT4 that might be a better potential therapeutic agent. While this grant application was under review, we worked with our collaborator Dr. Michael Rosenblum (MD Anderson Cancer Center) to complete a study comparing the cytotoxic effects of these two proteins on a panel of human cancer cell lines. These results, as well as additional findings showing that both GrB-TWEAK and GrB-Fc-IT4 administration can inhibit colon cancer and triple-negative breast cancer cell xenograft growth in immunodeficient nude mice, was published shortly after the start date of this DOD grant (6). Our *in vitro* data indicated that, in general, the GrB-Fc-IT4 fusion protein (shown in Fig. 1) was more potent than the GrB-TWEAK fusion protein in cancer cell cytotoxicity assays. Our results using various NSCLC cell lines are shown in Table 1, where GrB-Fc-IT4 had a higher targeting index (TI) in 5/9 cell lines. Additional research conducted just prior to and during this funding period has revealed that GrB-Fc-IT4 (i) binds to Fn14 in Biacore assays, (ii) is internalized by Fn14-expressing cancer cells, and (iii) primarily kills cancer cells via induction of programmed cell death (apoptosis) (Ref. 6 and data not shown).

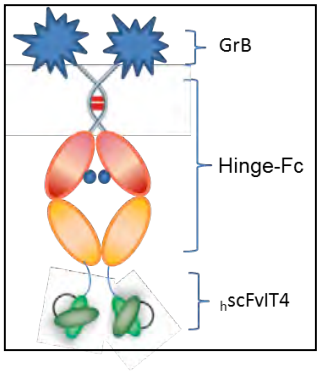


Figure 1. Schematic of GrB-Fc-IT4 fusion protein. GrB-Fc-IT4 is a homodimer in which an Fc domain of human IgG1 is covalently linked to GrB (N-terminus) and the anti-Fn14 humanized scFv of ITEM-4 (C-terminus). Also shown are the disulfide bridge of hinge and the approximate position of the N-linked oligosaccharides attached at Asn297 in the IgG1 Fc-domain.

Table 1.
Comparative IC_{50} values and targeting indices of the GrB-TWEAK and GrB-Fc-IT4 fusion constructs on various Fn14-positive NSCLC cell lines.

	IC_{50} (nmol/L)		Targeting index[a]		
Cell line	**GrB**	**GrB-TWEAK**	**GrB-Fc-IT4**	**GrB-TWEAK**	**GrB-Fc-IT4**
HCC827	1,406	97.2	114	14	12
HCC2279	525.7	50.4	18.5	10	28
H1437	>3,200	15.9	13.5	>201	>237
H1975	>3,200	13.8	114	>231	>28
A549	601.6	152.4	18.7	4	32
H358	>3,200	55.2	121	>58	>26
H2073	3,200	270	114	>12	>28
H3255	2,359	148.2	26.8	17	88
H520	259	56.7	61.7	5	4

[a]Targeting index represents IC_{50} of GrB/IC_{50} of GrB-based fusion proteins.

One of the other experiments we proposed in Aim 1 and completed this funding period was to test whether the Fn14-targeted constructs have cytotoxic effects on EGFR-mutant, tyrosine kinase inhibitor (TKI)-resistant NSCLC cells. To complete this task, we used H1975 cells, which have a secondary "gatekeeper" T790M resistance mutation, and HCC827 GR6 cells, which exhibit EGFR TKI resistance via activation of the c-Met and HER3 signaling pathways. We first conducted Western blot analysis to examine Fn14, phospho-EGFR, and phospho-Met levels in these two cell lines and several others as well. We found that the H1975 and HCC827 GR6 cells expressed high levels of Fn14 (Fig. 2). We then conducted dose-response assays using GrB (a control for cell-killing effects due exclusively to plasma membrane penetration), TWEAK-GrB and GrB-Fc-IT4. The H1975 data is shown in Table 1, where both Fn14-targeted constructs showed a respectable TI. The HCC827 GR6 data comparing the effects of GrB and GrB-Fc-IT4 is shown in Fig. 3. In these cells, the TI is at least 3.8. In consideration of these findings and our other *in vitro* results (Ref. 6 and data not shown), we intend to use Grb-Fc-IT4 as the agent of choice for *in vivo* work (see Aim 2 below).

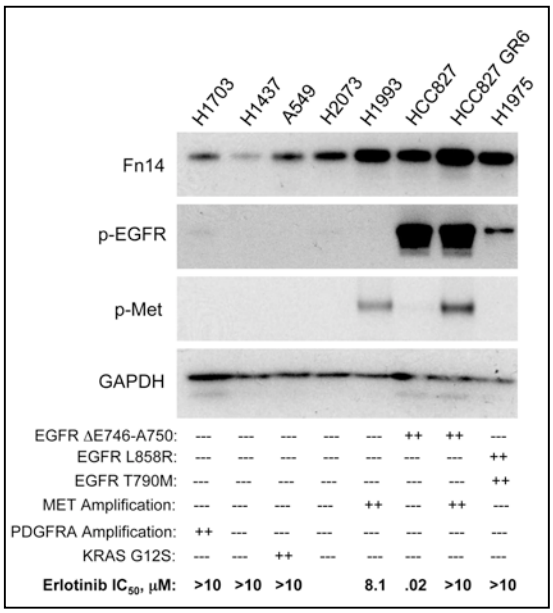


Figure 2. NSCLC cell lines with EGFR-activating mutations express high Fn14 levels. The indicated cell lines were harvested and cell lysates were prepared and immunoblotted with the indicated antibodies. Some of the known genetic characteristics of the cell lines as well as their sensitivity to the EGFR TKI erlotinib is provided on the bottom of the panel.

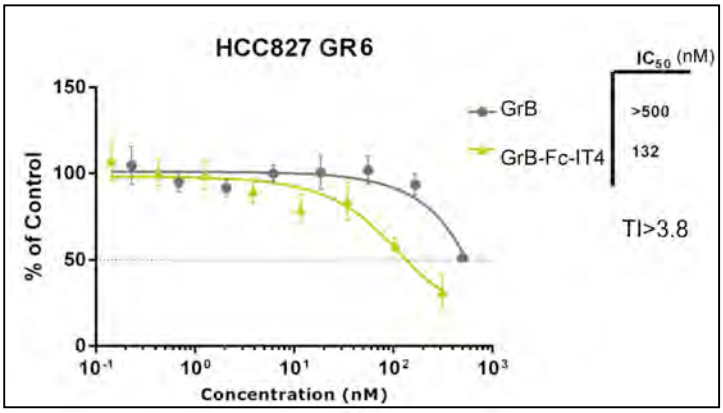


Figure 3. GrB-Fc-IT4 has cytotoxic activity when added to the EGFR TKI-resistant HCC827 GR6 NSCLC cell line. Cells were plated in quadruplicate in 96-well plates and allowed to adhere overnight. The cells were either left untreated or treated with various doses of purified GrB or GrB-Fc-IT4 and incubated for 72 hr. The remaining adherent cells were stained with crystal violet and solubilized with Sorenson's buffer. Absorbance was measured at 595 nm and plotted as percentage of untreated cells.

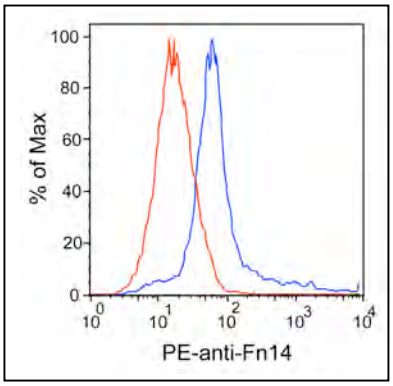
Specific Aim 2: To determine if GrB-TWEAK administration can inhibit NSCLC cell growth *in vivo* using both established cell line and patient-derived tumor tissue xenograft models.

MILESTONES- not yet completed
- Complete MTD experiment (Month 13)
- Complete xenograft assays using established NSCLC cell lines, including tissue analyses (Months 14-20)
- Complete xenograft assays using patient-derived NSCLC explants, including tissue analyses (Months 21-24)

In this Aim of the funded application we proposed to test whether our most potent Fn14-targeted cytotoxin was able to reduce lung cancer tumor growth *in vivo* using various NSCLC cell lines and NSCLC patient-derived explant tissue. In contrast to immortalized NSCLC cell lines like A549, these explants maintain the original properties of the human tumors since they are never placed into cell culture. It is generally established that patient-derived xenograft (PDX) studies such as the ones we proposed in the application may give the most information regarding the therapeutic potential of new biologics. Although we proposed to begin these studies in year 2, we have conducted some preliminary mouse work during this funding period (summarized below) to aid in the exact experimental design of the cytotoxin efficacy studies.

We stated in the application that all of our human NSCLC cell xenograft studies would be conducted using immunodeficient *nu/nu* athymic nude mice, which lack T cells. However, numerous groups use NOD-*scid*-common gamma-chain (NSG) mice for these types of studies because they lack mature T, B and NK cells. Since it is possible that NSG mice could exhibit more robust xenograft tumor establishment and more reproducible xenograft tumor growth kinetics, we are currently performing some comparative studies using these two mouse strains. In another group of experiments, we have conducted some preliminary work to ascertain if it would be possible to expand our Fn14-targeted cytotoxin *in vivo* work to include lung tumor subcutaneous growth studies in C57BL/6 mice, which have a normal immune system and thus are more representative of human cancer patients. For these experiments, we would need to use murine Lewis Lung Carcinoma (LLC) cells, which are syngeneic for the C57BL/6 mouse strain. We have shown by FACS analysis that LLC cells express Fn14 (Fig. 4) but we do not yet know if GrB-Fc-IT4 has cytotoxic activity on these cells. We have also confirmed that these cells form tumors when implanted subcutaneously in wild-type (WT) C57BL/6 mice, and tumor growth is more robust when the cells are mixed with the basement membrane extract Matrigel (Fig. 5). This LLC model could be quite informative because we could implant these cells into both WT and Fn14-knockout (KO) mice, administer GrB-Fc-IT4, and monitor on-target/off-tissue toxicity by measuring body weight and other parameters as stated in the application. We are currently maintaining a small colony of C57BL/6 WT, TWEAK-KO, and Fn14-KO mice in the event that we pursue this research direction.

Figure 4. Murine LLC cells express Fn14 on the cell surface. LLC were subjected to FACS analysis using PE-labeled anti-Fn14 mAb (blue line) or isotype control IgG (red line).



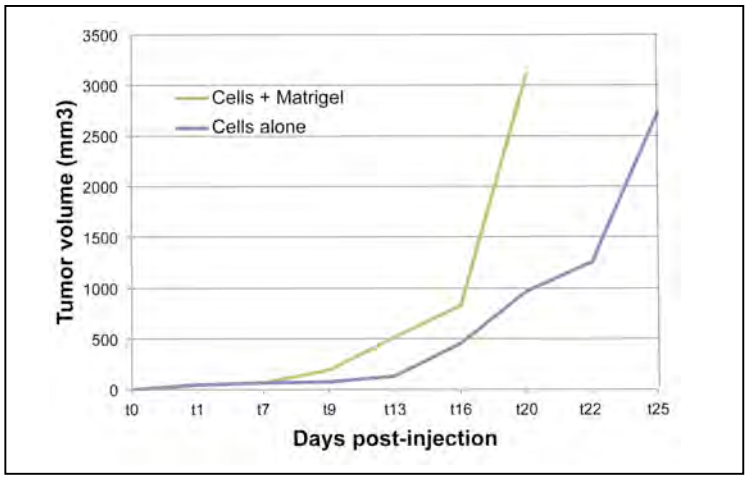


Figure 5. Murine LLC cell tumor growth in C57BL/6 mice. LLC cells (10^6) were resuspended in PBS and injected under the skin of 6-week old female C57BL/6 mice either alone (purple line) or in a 1:1 mixture with Matrigel (green line) (n=5 per group) Tumor size was measured 2-3 times per week with calipers. Tumor volume was calculated using the formula $V= (W/2)^2 \times L$.

b. What opportunities for training and professional development has the project provided?
Not applicable- nothing to report.

c. How were the results disseminated to communities of interest?
Not applicable- nothing to report.

d. What do you plan to do during the next reporting period to accomplish the goals?
We plan to conduct the cytotoxin *in vivo* efficacy studies essentially as we described in Aim 2 of the proposal. We will likely make some minor adjustments to the research strategy; for example, as mentioned earlier, we are now focusing on GrB-Fc-IT4 instead of TWEAK-GrB, we may use NSG mice instead of nude mice, and we may expand the Aim to include LLC cells and C57BL/6 mice. Also, in regard to the human NSCLC cell line xenograft experiments, we intend to design a "more elegant" approach. Specifically, we want to use lentivirus constructs to engineer a pair of KRas-mutated A549 cell lines. A549 cells will be infected with either a control short hairpin RNA (shRNA) or an Fn14 shRNA and stable cell lines will be generated by drug selection. The first cell line should express "normal" levels of Fn14 and the second should express reduced Fn14 levels. These cells will also be engineered to express firefly luciferase so we can non-invasively monitor tumor growth over time using the Xenogen bioluminescence imaging system. We will implant each cell line on a different side of ~6-8 nude mice and systemically administer either vehicle, GrB or GrB-Fc-IT4 as outlined in the proposal. Tumor growth will be measured and tumors retrieved and analyzed as outlined in the proposal.

4. Impact:

a. What was the impact on the development of the principal discipline(s) of the project?
Numerous groups are attempting to develop targeted toxins for use in cancer treatment. The work completed in Aim 1 demonstrating that a recombinant construct such as GrB-Fc-IT4 can bind the protein target and exhibit cytotoxic effects advances the field and thus has an impact on this research arena.

b. What was the impact on other disciplines?
Not applicable- nothing to report.

c. What was the impact on technology transfer?
If we obtain positive results in Aim 2, this research project could potentially initiate a dialog with biotechnology/pharmaceutical companies regarding additional studies to ascertain translatability to the clinic.

d. What was the impact on society beyond science and technology?
Not applicable- nothing to report.

5. Changes/Problems During the Reporting Period:

a. Changes in approach and reasons for change
We did initiate some mouse studies as the *in vitro* work was proceeding. As mentioned earlier, we originally thought we would begin these studies in year 2, but we felt it was necessary to begin some preliminary work during this reporting period.

b. Actual or anticipated problems or delays and actions or plans to resolve them
No problems or delays.

c. Changes that had a significant impact on expenditures
We did have mouse purchase and per diem charges this reporting period that were not in the original year 1 budget but we were under budget for our other supply costs so we did not exceed our year 1 direct costs budget.

d. Significant changes in use or care of human subjects
Not applicable.

e. Significant changes in use or care of vertebrate animals
All of the mouse strains and tumor growth studies mentioned above were approved by our IACUC:
IACUC protocol #0214020, last approval date 2/20/15
IACUC protocol #0414016, last approval date 4/17/15

f. Significant changes in use of biohazards and/or select agents
None.

6. Products:

a. Journal publications

Zhou, H., Mohamedali, K.A., Gonzalez-Angulo, A.M., Cao, Y., Migliorini, M., Cheung, L.H., LoBello, J., Lei, X., Qi, Y., Hittelman**,** W.N., **Winkles, J.A.,** Tran, N.L. and Rosenblum, M.G. (2014). Development of human serine protease-based therapeutics targeting Fn14 and identification of Fn14 as a new target overexpressed in TNBC. Mol Cancer Ther 13:2688-2705.

Perez, J.G., Tran, N.L., Rosenblum, M.G., Schneider, C.S., Connolly, N.P., Kim, A.J., Woodworth, G.F. and **Winkles, J.A.** (2015). The TWEAK receptor Fn14 is a potential cell surface portal for targeted delivery of glioblastoma therapeutics. Oncogene, advance online publication, 24 August 2015.

b. Website(s) or other Internet site(s)

Nothing to report.

c. Technologies or techniques

Nothing to report.

d. Inventions, patent applications, and/or licenses

Nothing to report.

e. Other Products

Nothing to report.

7. Participants & Other Collaborating Organizations:

a. What individuals have worked on the project?

Name:	Jeffrey A. Winkles
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.4
Contribution to Project:	Responsible for the direction, the design and conduct of all experiments
Funding Support:	

Name:	Rebeca Galisteo
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3.6
Contribution to Project:	Aim 1 studies and preliminary mouse work
Funding Support:	

Name:	Cheryl Armstrong
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6.0
Contribution to Project:	Aim 1 studies and preliminary mouse work
Funding Support:	

b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes. The PI of this DOD grant is listed as a co-PI at 10% effort on a recently funded 1 year grant (7/1/15-6/30/16) from the Elsa U. Pardee Foundation. This grant is focused on the use of drug-loaded nanoparticles for brain cancer treatment and has no scientific nor financial overlap with this funded DOD grant. This change in active support does not impact the PI's effort on the DOD grant.

c. What other organizations were involved as partners?

- **Organization Name:** Michael Rosenblum, Ph.D.; University of Texas MD Anderson Cancer Center
- **Location of Organization:** Houston, TX
- **Partner's Contribution to the projec**t*:*

  Collaboration – Our group has collaborated with Dr. Rosenblum's laboratory for the past 5 years on the development and testing of Fn14-targeted cancer therapeutics. During this funding period his laboratory received funds from this grant (via approved subcontract) to express and purify the various recombinant cytotoxins.

8. Special Reporting Requirements-N/A

9. References:

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4. Whitsett, T.G., Cheng, E., Inge, L., Asrani, K., Jameson, N.M., Hostetter, G., Weiss, G.J., Kingsley, C.B., Loftus, J.C., Bremner, R., Tran, N.L. and Winkles, J.A. (2012). Elevated expression of Fn14 in non-small cell lung cancer correlates with activated EGFR and promotes tumor cell migration and invasion. Am J Path 181:111-120.

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6. Zhou, H., Mohamedali, K.A., Gonzalez-Angulo, A.M., Cao, Y., Migliorini, M., Cheung, L.H., LoBello, J., Lei, X., Qi, Y., Hittelman**,** W.N., Winkles, J.A., Tran, N.L. and Rosenblum, M.G. (2014). Development of human serine protease-based therapeutics targeting Fn14 and identification of Fn14 as a new target overexpressed in TNBC. Mol Cancer Ther 13:2688-2705.

10. Appendices- Two published manuscripts are attached.

Published OnlineFirst September 19, 2014; DOI: 10.1158/1535-7163.MCT-14-0346

Large Molecule Therapeutics

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[1], Khalid A. Mohamedali[1], Ana Maria Gonzalez-Angulo[2,3], Yu Cao[1], Mary Migliorini[4], Lawrence H. Cheung[1], Janine LoBello[5], Xiudong Lei[6], Yuan Qi[7], Walter N. Hittelman[1], Jeffrey A. Winkles[4], Nhan L. Tran[8], and Michael G. Rosenblum[1]

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) tumor 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 hormone receptor–positive breast cancer, and in basal-like 2 tumors ($P = 0.01$) 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. *Mol Cancer Ther; 13(11); 2688–705. ©2014 AACR.*

[1]Department of Experimental Therapeutics, M.D. Anderson Cancer Center, Houston, Texas. [2]Department of Breast Medical Oncology, MD Anderson Cancer Center, Houston, Texas. [3]Department of Systems Biology, MD Anderson Cancer Center, Houston, Texas. [4]Department of Surgery, Center for Vascular and Inflammatory Diseases, and the Marlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland. [5]Integrated Cancer Genomics Division, Translational Genomics Research Institute, Phoenix, Arizona. [6]Department of Biostatistics, MD Anderson Cancer Center, Houston, Texas. [7]Department of Bioinformatics, MD Anderson Cancer Center, Houston, Texas. [8]Cancer and Cell Biology Division, Translational Genomics Research Institute, Phoenix, Arizona.

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

H. Zhou, K.A. Mohamedali, and A.M. Gonzalez-Angulo share first authorship of this article.

Current address for H. Zhou: Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, MN 55455; current address for Y. Cao: Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037.

Corresponding Author: Michael G. Rosenblum, Department of Experimental Therapeutics, M. D. Anderson Cancer Center, Unit 1950, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-792-3554; Fax: 713-745-9231; E-mail: mrosenbl@mdanderson.org

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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

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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 vasculature (14). In contrast, Fn14 expression in normal tissues is at relatively low levels. In cancer settings, overexpression 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 targeting 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.

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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 N-87, 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% CO_2 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 ($_h$scFvIT4; ref. 23). The mammalian expression vector pSecTag (Life Technologies) with a human IgGκ secretion leader sequence containing 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 proteins were expressed in HEK-293T cells and purified by immobilized metal affinity chromatography as described in Supplementary Methods. The leader sequence containing the $(His)_6$ tag was removed by exposure of the purified protein to enterokinase as described previously (8).

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 (33). Briefly, 5×10^5 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 fixed 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 ($\sim 3 \times 10^3$/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 targeting index (the ratio of IC_{50} for GrB vs. the IC_{50} 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. IC_{25}, IC_{50}, IC_{75}, 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

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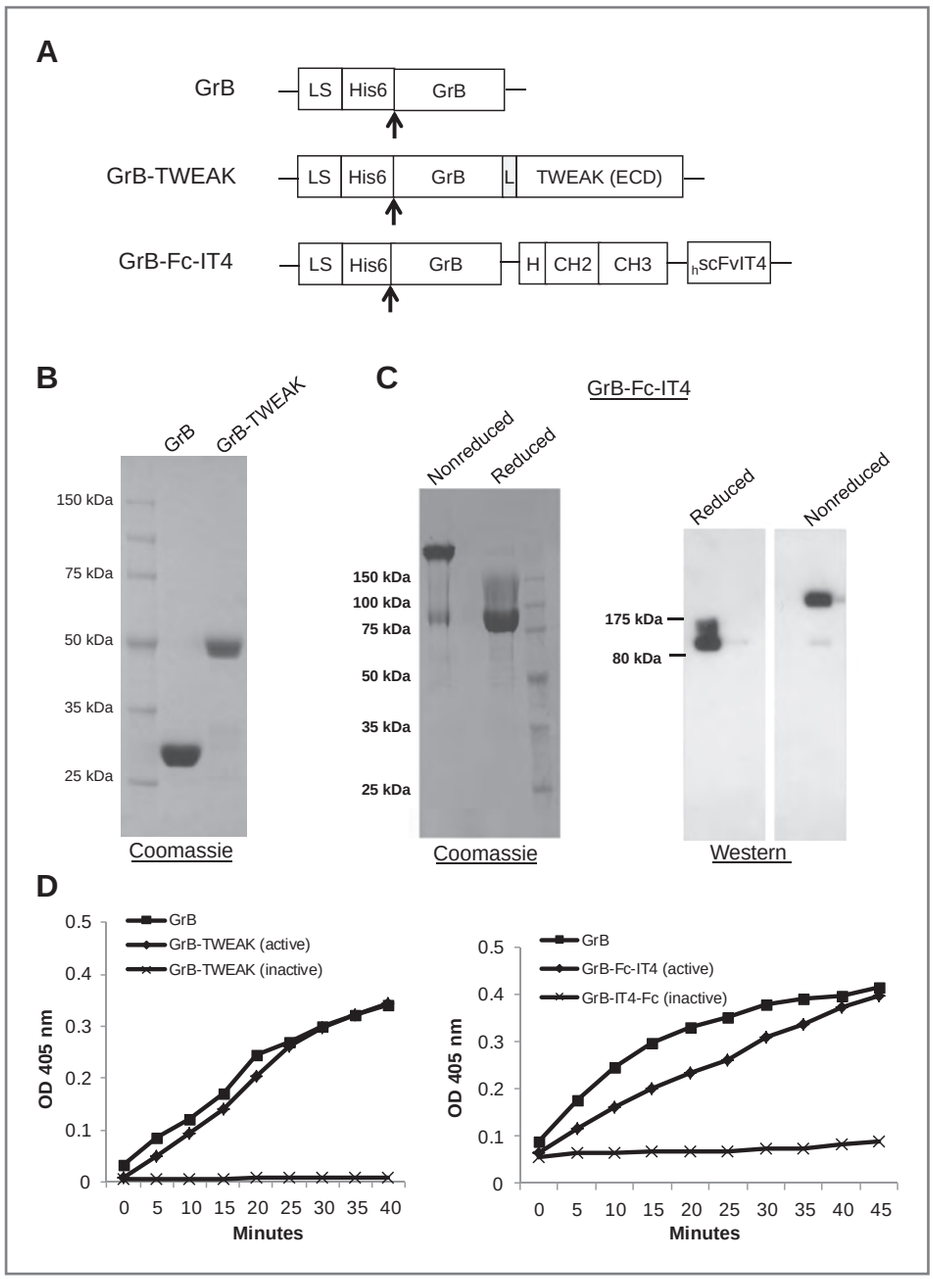

Figure 1. Preparation, purification, and enzymatic activity of GrB, GrB-TWEAK, and GrB-Fc-IT4 fusion proteins. A, schematic diagrams of the GrB, GrB-TWEAK, and GrB-Fc-IT4 constructs. The insert was cloned into the pSecTag vector and transiently expressed in HEK-293T cells. LS, the Igκ leader sequence 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-CH2-CH3, hinge CH2 and CH3 domains of a human IgG1 heavy chain; $_h$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.

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.

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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. The pellet was resuspended in 0.1 mL of mitochondrial extraction buffer and saved as the mitochondrial 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 8×10^6 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 mm^3, 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 (mm^3) ± SEM. Survival was calculated using a predefined cutoff volume of 400 mm^3 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 drug localization in MDA-MB-231/Luc tumors, female BALB/c nude mice were injected in the mammary fat pad with MDA-MB-231/Luc cells as described above. Two weeks later, mice were injected (i.v.) with GrB (200 μg), GrB-TWEAK (200 μg), or GrB-Fc-IT4 (200 μg). Twenty-four hours after injection, mice were sacrificed and tumor samples were collected 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.

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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/tnbc/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. Statistical analyses were carried out using SAS 9.2 (SAS Institute) and S-Plus 7.0 (Insightful Corporation).

For the laboratory data, statistical analyses were conducted using GraphPad Prism 5 with one-way ANOVA analysis, followed by Dunnet *t* test to compare the tumor volumes between the control- and drug-treated groups. Survival comparisons between groups were analyzed by log-rank test. Differences between groups were considered significant when the *P* value was ≤ 0.05.

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_ds) 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).

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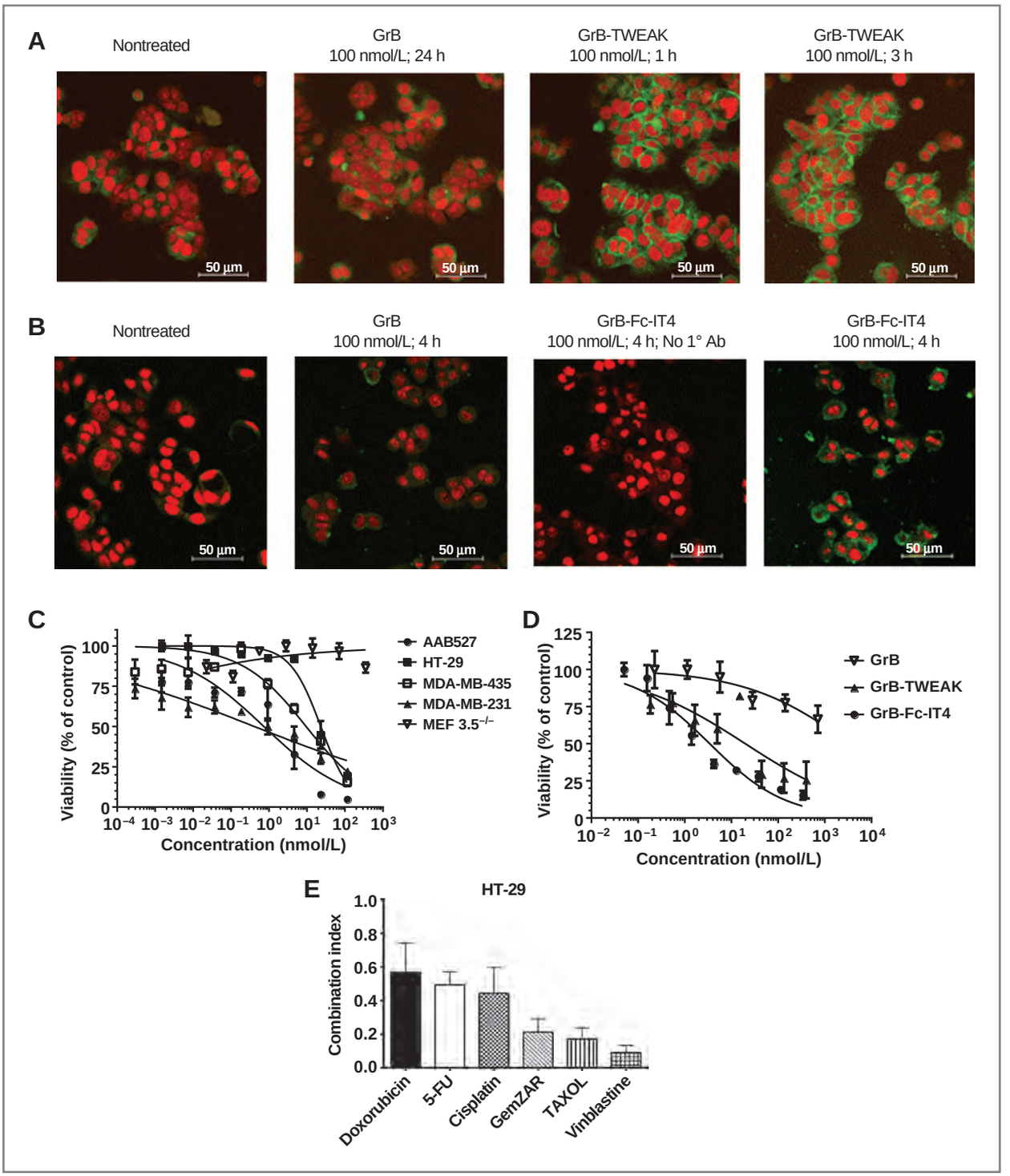

Figure 2. *In vitro* characterization of GrB-TWEAK and GrB-Fc-IT4 fusion proteins. GrB-TWEAK (A) and GrB-Fc-IT4 (B) specifically internalize into Fn14-expressing HT-29 cells. Cells were either left untreated or treated with 100 nmol/L GrB, GrB-TWEAK, or GrB-Fc-IT4 for the indicated times. The cells were 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.*)

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Table 1. Comparative IC_{50} values of the GrB-TWEAK and GrB-Fc-IT4 fusion constructs against various cancer cell lines

			IC_{50} (nmol/L)			Targeting index[a]	
Cell line	**Tumor type**	**Fn14**	**GrB**	**GrB-TWEAK**	**GrB-Fc-IT4**	**GrB-TWEAK**	**GrB-Fc-IT4**
AsPc-1	Pancreatic	++++	1,297	169.5	17.2	8	75
Capan-2	Pancreatic	++++	>3,200	39.9	20.7	>80	>155
Capan-1	Pancreatic	++	2,344	31.2	37.2	75	63
L3.6p1	Pancreatic	++	1,215	56	34.5	22	35
HCC827	Lung	++++	1,406	97.2	114	14	12
HCC2279	Lung	++++	525.7	50.4	18.5	10	28
H1437	Lung	+++	>3,200	15.9	13.5	>201	>237
H1975	Lung	+++	>3,200	13.8	114	>231	>28
A549	Lung	+++	601.6	152.4	18.7	4	32
H358	Lung	+++	>3,200	55.2	121	>58	>26
H2073	Lung	+	3,200	270	114	>12	>28
H3255	Lung	+	2,359	148.2	26.8	17	88
H520	Lung	+/−	259	56.7	61.7	5	4
WM35P2N1	Melanoma	++++	1,543	122.1	143.8	13	11
SB2	Melanoma	+++	>1,923	21.6	17	>89	>113
WM35	Melanoma	+	>1,923	68.4	3.1	>28	>620
A375	Melanoma	+	>1,923	226.6	73.1	>267	>26
SK-MEL-28	Melanoma	+/−	>1,923	519	284	>4	>7
MDA-MB-231	Breast	+++	>1,931	6.9	5.3	>279	>364
MDA-MB-231/Luc	Breast	+++	3,554	15.9	3.4	224	378
MCF-7	Breast	+	>1,923	61.5	34.7	>31	>55
HT-29	Colon	++++	3,200	51.9	23	>62	>139
MEF 3.5−/−	Mouse embryonic fibroblast	−−	3,200	>900	>900		

[a]Targeting index represents IC_{50} of GrB/IC_{50} of GrB-based fusion proteins.

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 IC_{50} 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

(*Continued.*) D, MDA-MB-231/Luc cells were treated with indicated concentration of GrB, GrB-TWEAK, or GrB-Fc-IT4 for 72 hours. Cytotoxicity was assessed by crystal violet staining as previously described (33). E, cytotoxicity of GrB-TWEAK when used in combination with various chemotherapeutic agents. Cells were treated with known cytotoxic doses of GrB-TWEAK in combination with fixed doses of various chemotherapeutic agents. GrB-TWEAK was administered 6 hours before the chemotherapeutic agent, 6 hours after administration of the chemotherapeutic agent, or coadministered. Cell viability was assessed after 72 hours. No difference in sensitivity to GrB-TWEAK was observed on the basis of the order of treatment. Data shown are from pretreatment of cells with chemotherapeutic agents before addition of GrB-TWEAK. Mean CIs were calculated using Calcusyn software and synergistic values are plotted. CI = 1 indicates an additive effect, CI > 1 indicates antagonism, and CI < 1 indicates synergism. Thus, GrB-TWEAK in combination with various chemotherapeutic agents shows synergistic effects on HT-29 cells.

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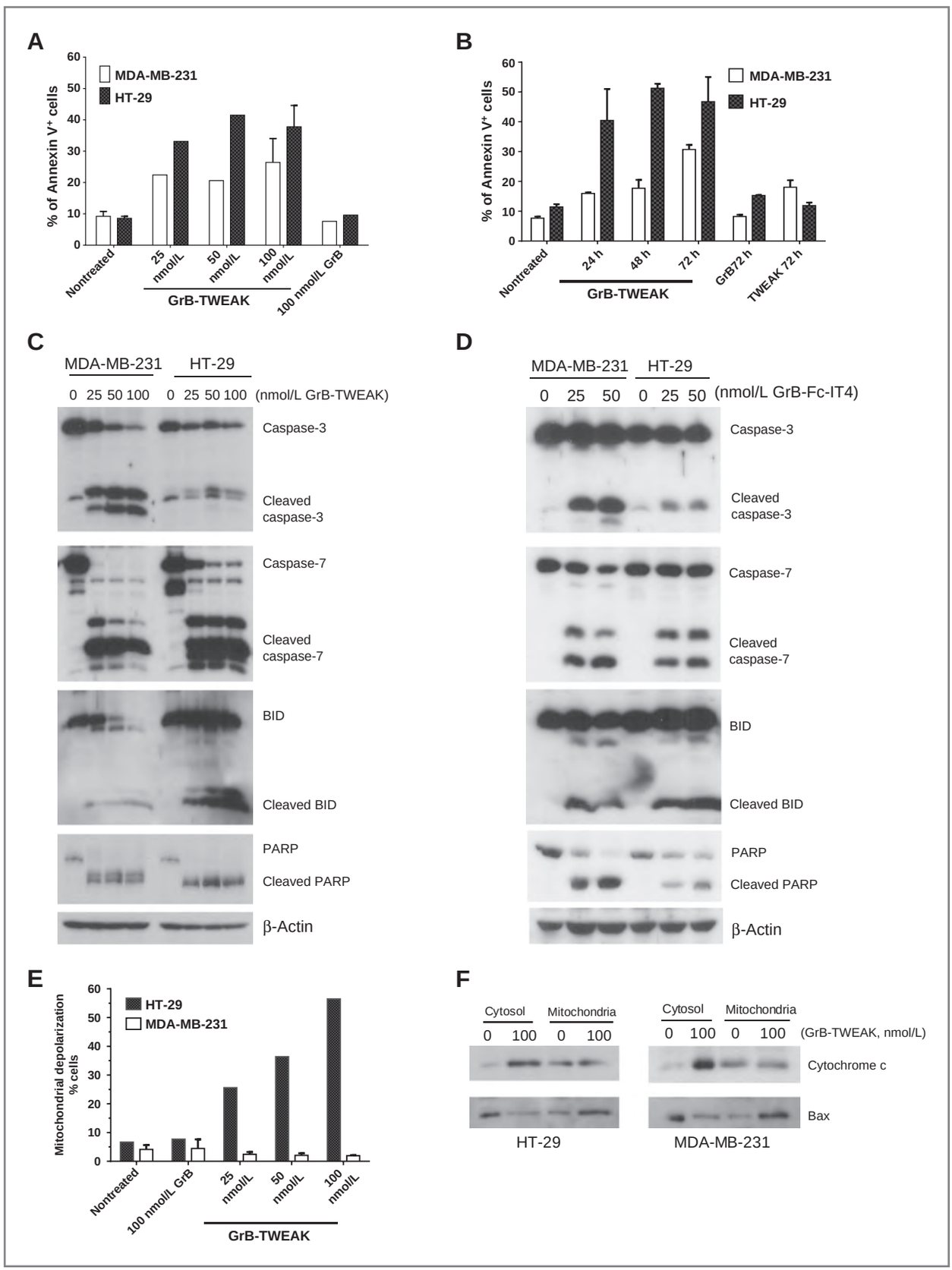

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expression (such as MEF3.5−/− and Jurkat; Table 1 and Supplementary Table S1) were not specifically targeted by the fusion proteins. As shown in Table 1 and Supplementary Table S1, melanoma was generally found to be the most sensitive cancer type. 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 IC_{50} 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). IC_{50} 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 cancer 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). Interestingly, 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-fmk (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 IC_{50} 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

Figure 3. GrB-based constructs induce apoptosis in HT-29 and MDA-MB-231 cells. A, cells were either left untreated or treated with the indicated concentration of GrB or GrB-TWEAK for 24 hours and then stained with Alexa Fluor 488 Annexin V and PI, followed by flow-cytometric analysis. The percentage of Annexin V^+ cells includes cells in early (Annexin V^+ PI^-) and late (Annexin V^+ PI^+) apoptosis. B, cells were either left untreated or treated with 100 nmol/L GrB-TWEAK for the indicated times or treated with 100 nmol/L GrB or TWEAK for 72 hours, and then stained with Alexa Fluor 488 Annexin V and PI, followed by flow-cytometric analysis. The percentage of Annexin V^+ cells includes cells in early (Annexin V^+ PI^-) and late (Annexin V^+ PI^+) apoptosis. C, cells were left untreated or treated with indicated concentration of GrB-TWEAK for 24 hours. Cells were collected and whole-cell lysates were analyzed by Western blot analysis with the indicated antibodies. D, cells were either left untreated or treated with the indicated concentration of GrB-Fc-IT4 for 24 hours. Cells were collected and whole-cell lysates were analyzed by Western blot analysis with the indicated antibodies. E, cells were left untreated or treated with 100 nmol/L GrB or the indicated concentration of GrB-TWEAK for 72 hours and then assayed for mitochondrial membrane depolarization by flow-cytometric analysis. F, cells were either left untreated or treated with 100 nmol/L GrB-TWEAK for 24 hours and then the cytosolic and mitochondrial fractions were isolated. Fractions were analyzed by Western blot analysis using anti-cytochrome c and anti-Bax antibodies.

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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/LCC6^{MDR1} 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/LCC6^{MDR1} 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 IC_{50}s of GrB-TWEAK and GrB-Fc-IT4 on the MDR cells were similar to their parental cells (less than $\sim$ 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

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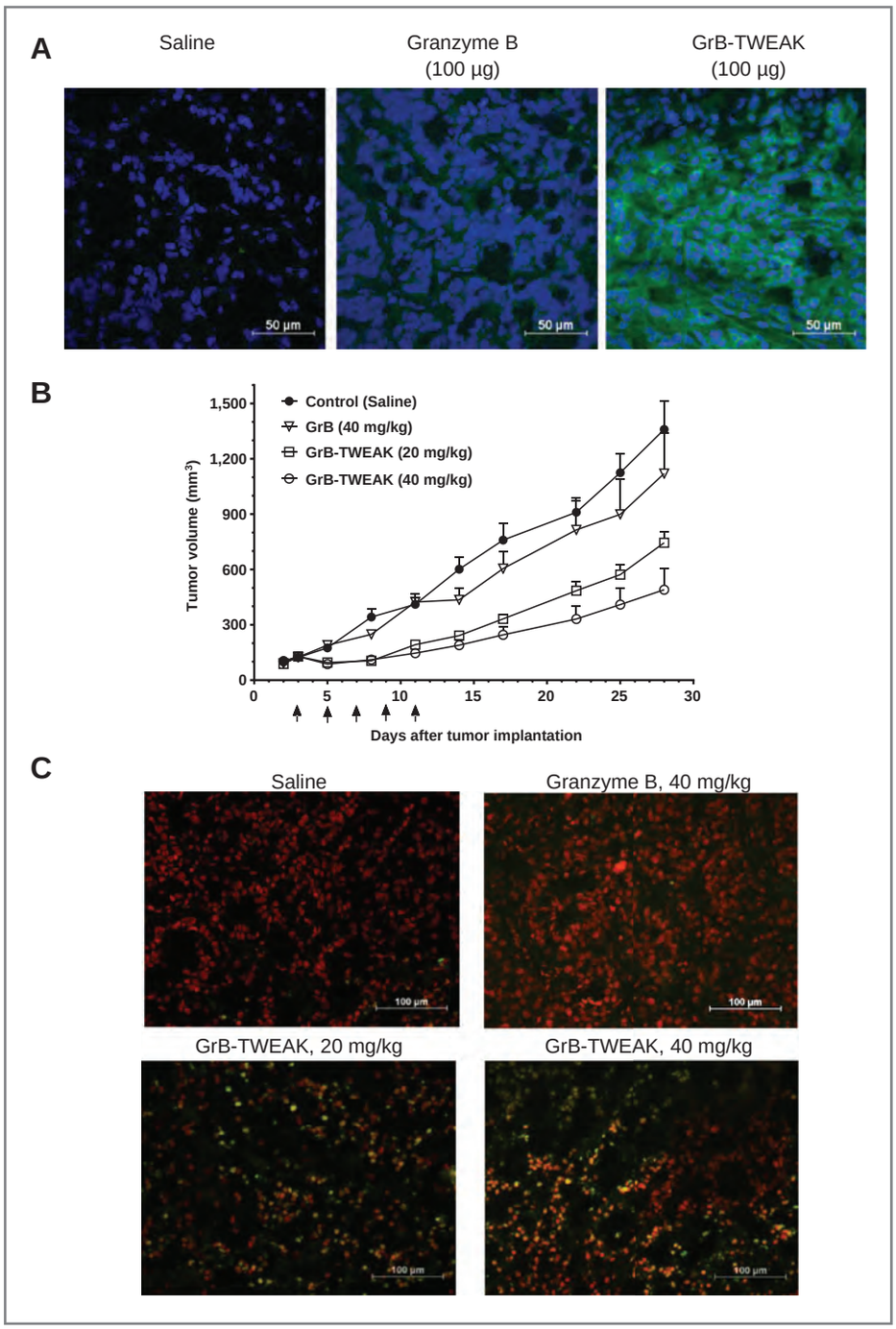

Figure 4. Specific localization and antitumor efficacy of GrB-TWEAK in HT-29 tumors. A, upon termination of the efficacy study, tumor tissues were removed, fixed, and assayed for the presence of GrB by immunofluorescence using a rabbit anti-GrB antibody (green). The cells were counterstained with DAPI (blue) to identify nuclei. B, HT-29 cells were injected subcutaneously into mice and tumors were allowed to grow to approximately 150 mm^3 in volume. Groups ($n = 5$) were injected (i.v., tail vein) with either saline (control), GrB, or GrB-TWEAK at 20 mg/kg or 40 mg/kg total dose. Data represent mean tumor volumes ± SEM, and arrows indicate dosing days. C, apoptosis detection in tumor tissue. Tumor tissue sections were stained by TUNEL (green) and the cells were counterstained with propidium iodide (red) to identify nuclei. The sections were analyzed using a Nikon Eclipse TS 100 fluorescence microscope.

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

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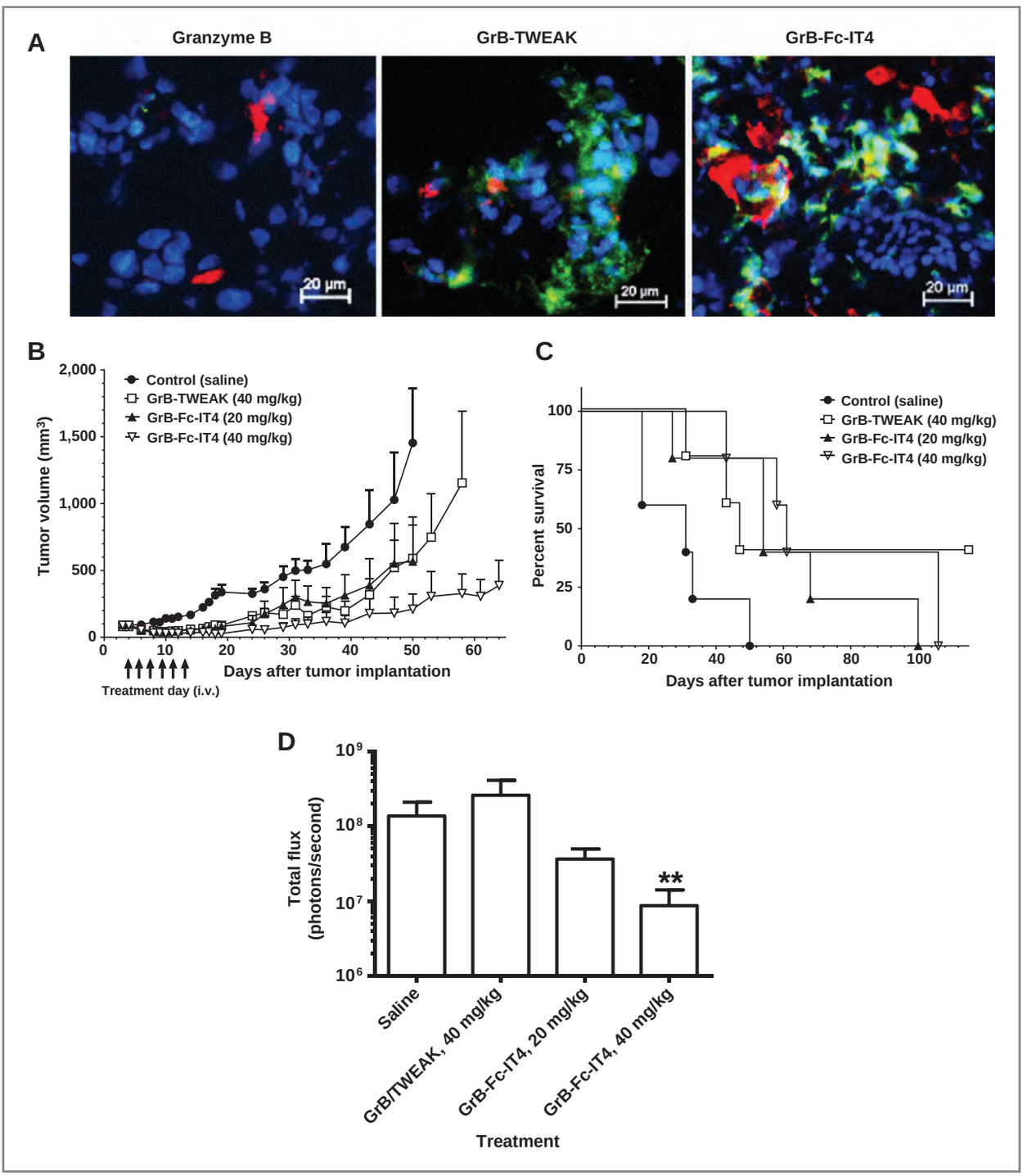

Figure 5. *In vivo* activity of GrB-TWEAK and GrB-Fc-IT4 in an MDA-MB-231/Luc breast tumor orthotopic xenograft model. A, GrB, GrB-TWEAK, or GrB-Fc-IT4 was administered (i.v.) to MDA-MB-231/Luc tumor-bearing mice. One day later, animals were sacrificed, and tumor tissues were removed, fixed, and stained 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 mm^3. Tumor size was assessed by direct caliper measurement. Efficacy data are plotted as mean tumor volume (in mm^3) ± 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.

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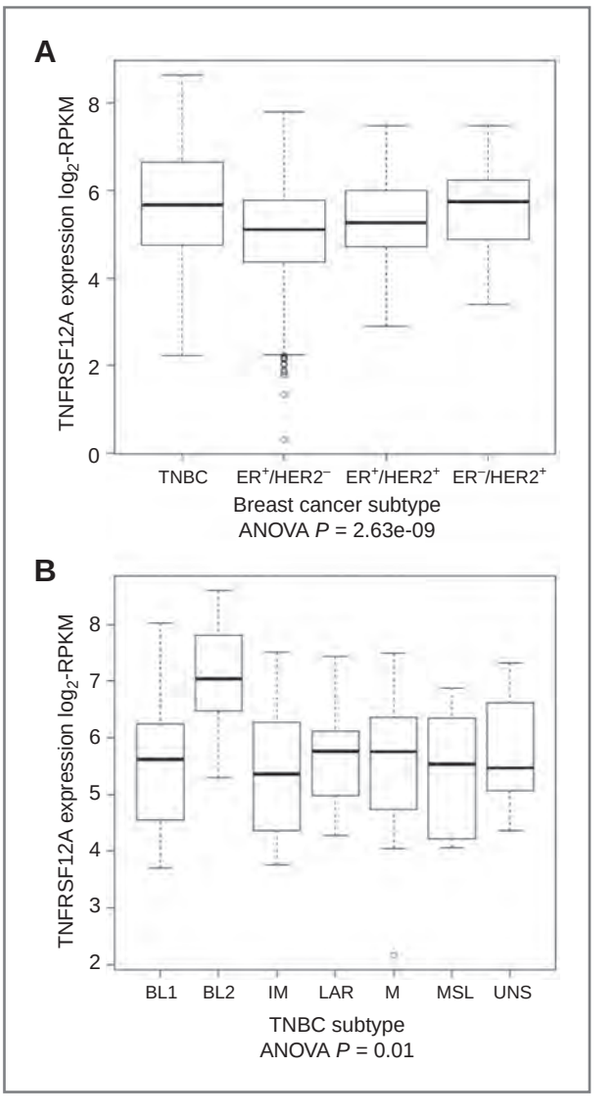

Figure 6. Analysis of Fn14 (TNFRSF12A) gene expression in breast cancer. A, according to breast cancer molecular subtype. B, according to TNBC subtype. Log_2-transformed RPKM, reads per kilobase per million mapped reads.

($n = 100$). Figure 6A 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%

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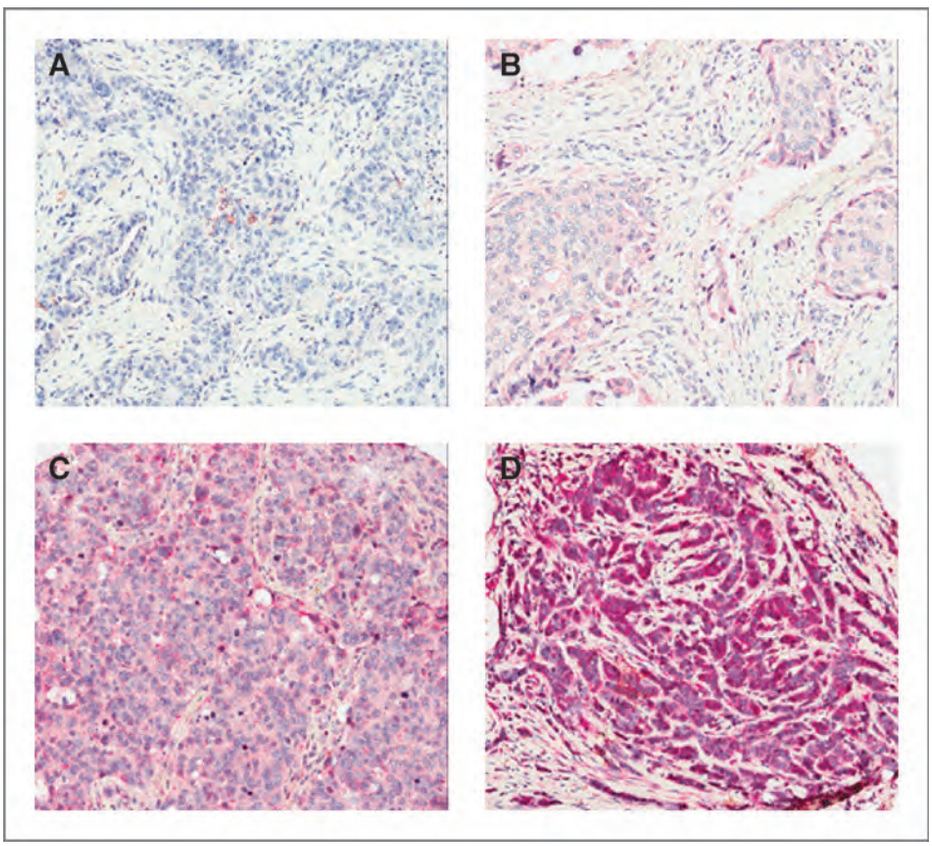

Figure 7. Fn14 IHC expression: A, negative 0+; B, negative 1+; C, positive 2+; D, positive: 3+.

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 cells 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

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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 $_{h}$scFvIT4 is fused to the carboxy terminus of the Fc domain of the IgG (Supplementary Fig. S8). By fusing $_{h}$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 *in vivo* efficacy that may be partially mediated by ADCC and activation of NF-κB signaling even though neither antibody showed significant cytotoxicity *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 *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 *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 *in vitro* and *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.

Authors' Contributions

Conception and design: H. Zhou, K.A. Mohamedali,A.M. Gonzalez-Angulo, L.H. Cheung, J.A. Winkles, N.L. Tran, M.G. Rosenblum

Development of methodology: H. Zhou, A.M. Gonzalez-Angulo, Y. Cao, M. Migliorini, L.H. Cheung, W.N. Hittelman

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Zhou, K.A. Mohamedali, A.M. Gonzalez-Angulo, M. Migliorini, L.H. Cheung, J. LoBello, W.N. Hittelman

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Zhou, K.A. Mohamedali, A.M. Gonzalez-Angulo, L.H. Cheung, J. LoBello, X. Lei, Y. Qi, W.N. Hittelman, N.L. Tran, M.G. Rosenblum

Writing, review, and/or revision of the manuscript: H. Zhou, K.A. Mohamedali, A.M. Gonzalez-Angulo, L.H. Cheung, J. LoBello, X. Lei, W.N. Hittelman, J.A. Winkles, N.L. Tran, M.G. Rosenblum

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Zhou, A.M. Gonzalez-Angulo, Y. Cao
Study supervision: H. Zhou, L.H. Cheung, M.G. Rosenblum

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REVIEW

The TWEAK receptor Fn14 is a potential cell surface portal for targeted delivery of glioblastoma therapeutics

JG Perez[1], NL Tran[2], MG Rosenblum[3], CS Schneider[1], NP Connolly[1], AJ Kim[1,4,5], GF Woodworth[1,5] and JA Winkles[5,6,7]

Fibroblast growth factor-inducible 14 (Fn14; TNFRSF12A) is the cell surface receptor for the tumor necrosis factor (TNF) family member TNF-like weak inducer of apoptosis (TWEAK). The *Fn14* gene is normally expressed at low levels in healthy tissues but expression is significantly increased after tissue injury and in many solid tumor types, including glioblastoma (GB; formerly referred to as 'GB multiforme'). GB is the most common and aggressive primary malignant brain tumor and the current standard-of-care therapeutic regimen has a relatively small impact on patient survival, primarily because glioma cells have an inherent propensity to invade into normal brain parenchyma, which invariably leads to tumor recurrence and patient death. Despite major, concerted efforts to find new treatments, a new GB therapeutic that improves survival has not been introduced since 2005. In this review article, we summarize studies indicating that (i) *Fn14* gene expression is low in normal brain tissue but is upregulated in advanced brain cancers and, in particular, in GB tumors exhibiting the mesenchymal molecular subtype; (ii) Fn14 expression can be detected in glioma cells residing in both the tumor core and invasive rim regions, with the maximal levels found in the invading glioma cells located within normal brain tissue; and (iii) TWEAK:Fn14 engagement as well as Fn14 overexpression can stimulate glioma cell migration, invasion and resistance to chemotherapeutic agents *in vitro*. We also discuss two new therapeutic platforms that are currently in development that leverage Fn14 overexpression in GB tumors as a way to deliver cytotoxic agents to the glioma cells remaining after surgical resection while sparing normal healthy brain cells.

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INTRODUCTION

The TWEAK-Fn14 cytokine-receptor axis

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK), a member of the TNF superfamily,[1] acts on cells via binding to fibroblast growth factor-inducible 14 (Fn14), a 102-aa type I transmembrane protein that was initially discovered as a growth factor-inducible gene product[2,3] and then subsequently identified as the TWEAK receptor by Wiley *et al.*[4] TWEAK is initially synthesized as a type II transmembrane protein but it can be cleaved by furin to generate a soluble cytokine.[1,5] Both the membrane-anchored and soluble TWEAK isoforms can bind to Fn14.[5,6] TWEAK:Fn14 engagement promotes Fn14 trimerization and TNF receptor-associated factor binding to the Fn14 cytoplasmic tail,[7] which activates a number of intracellular signal transduction cascades (for example, the nuclear factor-κB (NF-κB) pathway). This can result in increased cell proliferation, survival, migration, differentiation or death, depending on the cellular context (Figure 1).[8–10] In addition, TWEAK is a pro-inflammatory[11,12] and pro-angiogenic[13,14] cytokine *in vivo*.

The *TWEAK* and *Fn14* genes are expressed at low levels in most healthy normal tissues but increased expression has been noted after tissue injury[8–10] and in many solid primary tumor types and tumor metastases. For example, TWEAK is highly expressed in liver,[15] ovarian,[16] colorectal,[14,17] bladder,[18] esophageal,[19] pancreatic[19] and prostate[20] cancers, as well as a high percentage of prostate cancer metastases.[20] Fn14 overexpression has been detected in over a dozen solid tumor types,[21,22] including the tumor types mentioned above[3,16,18–20,23,24], as well as brain cancer (see below),[25,26] breast cancer,[27–30] lung cancer[31,32] and melanoma.[33] It has also been reported that Fn14 is highly expressed in the metastatic lesions of breast,[28] colorectal,[21] melanoma,[33] non-small cell lung[34] and prostate[20] cancer patients.

Studies using TWEAK-null mice, Fn14-null mice or anti-TWEAK-neutralizing monoclonal antibodies (mAbs) have revealed that transient TWEAK/Fn14 signaling after acute tissue injury is critical for efficient wound repair.[8–10] However, chronic, dysregulated Fn14 activation has been implicated in the pathophysiology of several prominent human diseases, including cancer, cardiovascular disease, rheumatoid arthritis and inflammatory bowel diseases.[8–10,35–38] Accordingly, a number of TWEAK- or Fn14-targeted therapeutic agents are presently in preclinical development[35,36] and some agents have progressed into early-phase clinical trials.[22,39]

Glioblastoma

Brain cancer is a biologically and clinically diverse group of intracranial tumors, with most primary intrinsic tumors arising from glial cells (usually astrocytes or oligodendrocytes).[40,41]

[1]Department of Neurosurgery, University of Maryland School of Medicine, Baltimore, MD, USA; [2]Cancer and Cell Biology Division, Translational Genomics Research Institute, Phoenix, AZ, USA; [3]Department of Experimental Therapeutics, MD Anderson Cancer Center, Houston, TX, USA; [4]Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, MD, USA; [5]Marlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD, USA; [6]Department of Surgery, University of Maryland School of Medicine, Baltimore, MD, USA and [7]Center for Vascular and Inflammatory Diseases, University of Maryland School of Medicine, Baltimore, MD, USA. Correspondence: Dr JA Winkles, Department of Surgery, University of Maryland School of Medicine, UMB BioPark Building One, Room 320, 800 West Baltimore Street, Baltimore, MD 21201, USA.
E-mail: jwinkles@som.umaryland.edu
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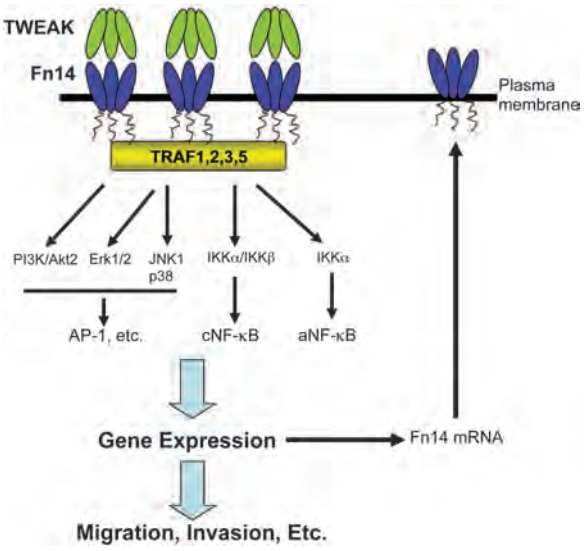


Figure 1. The TWEAK/Fn14 signaling dyad. Both membrane-anchored TWEAK and soluble TWEAK monomers assemble into trimers. TWEAK binding to the Fn14 extracellular domain promotes Fn14 trimerization and binding of TNF receptor-associated factor (TRAF) adaptor proteins to the Fn14 cytoplasmic tail. This interaction triggers the activation of various downstream protein kinases, only some of which are shown here, and signaling cascades, including the classical (c) and alternative (a) NF-κB pathways, resulting in transcription factor binding to genomic DNA and the expression of numerous TWEAK-inducible genes, including *Fn14*.[25,178] TWEAK: Fn14 engagement stimulates various cellular responses such as proliferation, migration, invasion and differentiation.

Glial cell tumors, collectively referred to as gliomas, represent ~80% of all malignant brain and central nervous system tumors, and glioblastoma (GB; World Health Organization grade IV glioma)[42] is the most common and lethal primary malignant brain tumor, with ~10 000 new cases in the United States per year.[43,44] GB tumors are characterized by extensive vascularization, a high mitotic index, cellular pleomorphism, genomic instability and tissue necrosis.[43] Technological advances during the past decade have allowed investigators to interrogate hundreds of GB surgical specimens at the molecular level using genomic, transcriptomic, epigenomic and proteomic analyses.[41,45–47] This research has catalogued the most common genetic alterations associated with this cancer type (that is, gene mutations, amplifications and deletions), thereby revealing several constitutively activated receptor tyrosine kinases and intracellular signaling nodes that may be targeted by precision therapeutics.[41,45,48] These studies and others have also demonstrated that although GB tumors exhibit significant intra-tumor molecular heterogeneity,[49–53] they can be classified into four major subtypes—proneural, neural, classical and mesenchymal—by whole-genome sequencing and expression profiling of surgically resected samples.[41,45,47,48,50,54] Each GB subtype is defined by a common genomic landscape, transcriptional profile, disease pathophysiology and patient prognosis.[41,45,47,48,50,54]

The current standard-of-care for patients with GB consists of maximal surgical resection or biopsy followed by high-dose radiation and concomitant oral chemotherapy using the DNA-alkylating agent temozolomide (brand name Temodar),[55] which can cross the blood–brain barrier (BBB) because of its small size and lipophilic properties. Despite this multimodal course of treatment, the median overall survival of GB patients is ~15 months after diagnosis,[55,56] with ~95% of patients succumbing to this disease within 5 years.[44] Patients whose tumors exhibit epigenetic silencing of the DNA repair enzyme O^6-methylguanine-DNA methyltransferase experience the best treatment outcomes;[56–58] however, disease progression occurs in all patients. One of the primary reasons for poor patient prognosis is that glioma cells are highly invasive; consequently, there is extensive tumor cell infiltration into surrounding healthy brain tissue.[59–61] These invading cells, which can be found centimeters away from the main tumor mass, cannot be removed by surgical resection of the tumor core or safely targeted by adjuvant radiotherapy because of the potential to harm nearby normal neurons and glial cells. Glioma cells residing in tumor margins are also relatively resistant to chemotherapeutics, primarily due to the BBB;[62] hence, these cells frequently transition from an invasive to a proliferative state, which leads to tumor recurrence, brain injury and patient death. Thus, novel therapies that target the invasive rim cells left behind after surgical resection are sorely needed in order to significantly improve the survival of GB patients. This is a challenging problem, as there are several critical barriers to effective delivery of GB therapeutics (see below). These barriers include the following: (i) the BBB; (ii) the electrostatically charged and anisotropic extracellular space found between brain cells, which contains extracellular matrix (ECM) proteins and proteoglycans; and (iii) the glialymphatic system,[63] a paravascular, pseudo-lymphatic drainage pathway that can act as a sink for small molecule drugs, gene therapy constructs and protein-based therapeutics.[64,65]

Challenges to GB patient therapy

The location of GB tumors presents several unique barriers to systemic drug treatment.[64,65] First, the BBB, consisting of cerebral endothelial cells connected together by tight junctions, a thick basement membrane, and astrocytic end-feet, controls the passage of most molecules and drugs from the blood circulation to the brain.[62,66] It has been estimated that >98% of small-molecule drugs and nearly all biologics (for example, therapeutic mAbs) do not cross the BBB.[67] Although this barrier is altered in the hypoxic core region of advanced brain tumors,[68] unresectable, invading tumor cells are consistently found in brain regions exhibiting a relatively intact BBB.[69–71] As mentioned above, these invading tumor cells are difficult to treat, owing to their close proximity to functioning brain cells, and contribute to tumor recurrence. Second, the anisotropic, electrostatically charged extracellular space found between brain cells, which contains a dense network of ECM components,[72,73] comprises 10–20% of total brain volume and acts as a 'brain penetration barrier' for both systemic and local drug delivery.[74,75] It has been suggested that the extracellular space in brain tumors may constitute a larger percentage of the total brain volume compared with the healthy brain extracellular space, with a more complex, tortuous structure and smaller pores.[76] Several strategies have been proposed to transiently disrupt the BBB to improve the delivery of therapeutic agents to GB tumors (for example, intra-arterial mannitol and focused ultrasound), but each of these methods have limitations that must be overcome before widespread clinical use.[62,64,77]

In consideration of the challenges associated with systemic drug administration, there is great interest in applying local drug delivery strategies for the treatment of malignant gliomas.[64,65,78] Gliadel (Arbor Pharmaceuticals, Atlanta, GA, USA) is a Food and Drug Administration-approved, biodegradable, polyanhydride-based polymer wafer containing the alkylating agent carmustine (*N*,*N*′-bis-[2-chloroethyl]-*N*-nitrosurea), a chemotherapeutic that exhibits high hematologic cell toxicity when administered systemically.[78–80] These wafers, which are ~1.5 cm in diameter, are implanted into the surgical resection cavity following tumor excision. Gliadel wafer implantation produces a small but significant improvement in median patient survival time compared with placebo wafers (~2 months).[78,79,81] For this form of interstitial chemotherapy, drug delivery is mediated by diffusion; consequently, Gliadel efficacy is likely limited, at least in part, by

the inability to achieve therapeutic drug concentrations more than a few millimeters beyond the immediate tumor resection margin.[79,82]

Another local drug delivery technique that has been used for GB patients is convection-enhanced delivery (CED), which is a hydrostatic and osmotic pressure-driven infusion method to generate bulk flow of therapeutic agents through the brain interstitial spaces.[65,78,83] Animal studies and human clinical trials have been carried out using CED as a means to deliver various anti-cancer agents including conventional chemotherapeutics, nanoparticles (NPs), mAbs and targeted toxins to malignant glioma cells.[84] Several limitations to the success of this approach were noted in the early GB CED trials; for example, catheter placement can greatly effect drug delivery[85–88] and there is a certain degree of drug backflow along the catheter.[78,89] In addition, even with CED, there is poor drug penetration to the infiltrative glioma cells residing in the invasive rim region (the brain area at risk for tumor recurrence).[88] Accordingly, efforts are underway to optimize the CED approach, including the use of real-time imaging techniques to assess the best catheter positioning and infusion parmeters for effective drug distribution.[90–93] In summary, for both systemic and local drug delivery methods, the limited penetration of therapeutic agents into tumor cell-infiltrated normal brain tissue remains a key hurdle to increasing the survival of GB patients.

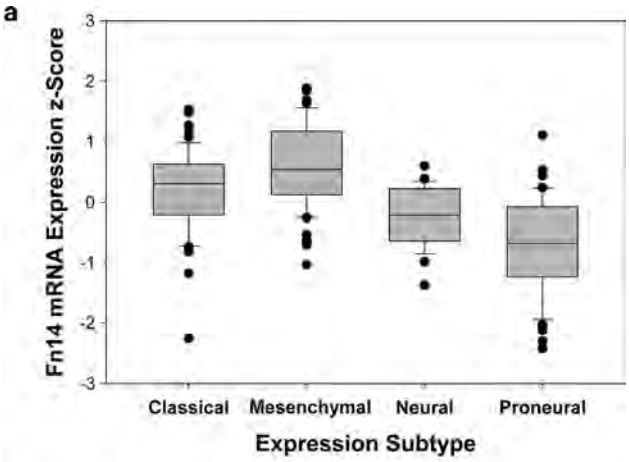
THE TWEAK/FN14 AXIS AND GB

Fn14 gene expression is upregulated in advanced brain cancers and, in particular, in GB tumors exhibiting the mesenchymal molecular subtype

The first study reporting that *Fn14* gene expression was elevated in GB samples compared with normal brain tissue was published by Tran *et al.* in 2003.[26] Fn14 mRNA levels were analyzed by real-time quantitative PCR in 3 normal brain samples, 1 oligodendroglioma, 2 pilocytic astrocytomas, 2 low-grade astrocytomas, 4 anaplastic astrocytomas and 16 GBs. The highest expression of Fn14 mRNA was observed in the GB specimens, with ~70% of the samples showing fivefold induction over normal brain tissue. Subsequent gene expression profiling experiments using larger sample numbers have confirmed Fn14 mRNA levels are low in normal brain and increase with glioma grade.[25,94,95] In addition, it has been reported that Fn14 mRNA expression levels in GB tumors inversely correlate with patient survival.[25] Finally, Fn14 is one of the 37 genes upregulated in the poor prognosis group of a 58-gene signature that is a strong prognostic predictor for patients diagnosed with oligodendroglial or astrocytic tumors, including GB.[96]

Elevated Fn14 expression in the GB tumor core has also been observed at the protein level using immunohistochemistry (IHC).[25,26,97] In one report, Fn14 expression was examined using a brain tumor tissue microarray and an Fn14-specific mAb, and the stain intensity was scored as negative, weak, moderate or strong.[25] All 9 of the non-neoplastic brain specimens had no staining or weak staining; in contrast, only 4 of the 27 GB specimens were scored negative or weak, whereas the other 23 specimens exhibited moderate or strong staining.

We recently interrogated The Cancer Genome Atlas GB tumor data set to examine Fn14 mRNA expression levels in 195 tumors representing the 4 different GB molecular subtypes that were mentioned above. This analysis revealed that *Fn14* gene expression is most frequently upregulated in mesenchymal subtype tumors (Figure 2). Mesenchymal subtype tumors frequently exhibit constitutive NF-κB[98] and STAT3[99] activation; thus, this finding is consistent with studies indicating that *Fn14* is an NF-κB- and STAT3/5-regulated gene (Tran, unpublished data).[25,100] Tumors with the mesenchymal gene expression signature are highly aggressive, invasive tumors that primarily arise *de novo*, and patients harboring these tumors have the poorest prognosis.[41,45,47,48,50,54] Interestingly, some proneural subtype GB tumors, on recurrence, tend to shift toward the mesenchymal subtype, and Phillips *et al.*[54] have reported that Fn14 mRNA expression is significantly upregulated (~2.4-fold increase) in those recurrent tumors where this phenotypic shift is detected.



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	Classical	Mesenchymal	Neural	Proneural
Classical		0.0036	0.0038	< 0.0001
Mesenchymal	0.0036		< 0.0001	< 0.0001
Neural	0.0038	< 0.0001		0.0031
Proneural	< 0.0001	< 0.0001	0.0031	

Figure 2. Analysis of Fn14 mRNA expression in GB molecular subtypes. (**a**) Fn14 (queried as TNFRSF12A) mRNA expression data in 195 GB tumors was downloaded as *Z*-scores from cbioportal.org.[179,180] *Z*-scores were computed by cbioportal.org relative to the expression distribution of each gene in tumors that are diploid for this gene. Patients used in this analysis were limited to the provisional The Cancer Genome Atlas (TCGA) data set.[46] The provisional data set using the Affymetrix U113 microarray platform is the only GB data set with annotation for classical, mesenchymal, neural and proneural expression clusters on cbioportal.org. Patient/sample sets for each expression cluster were plotted as box and whisker plots. Classical $n = 54$, mesenchymal $n = 56$, neural $n = 29$ and proneural $n = 56$. Whiskers of the plot map the maximum and minimum *Z*-score for each expression cluster. Bar in box represents the mean value for the Fn14 *Z*-score of each group. Top and bottoms of the box represent the 25th and 75th percentile, respectively, of the Fn14 *Z*-score values for each group. **(b)** Table showing *P*-values for significance of mean across GB subtypes for Fn14 expression. Each of the mean expression values are significantly different from one another ($P < 0.05$) as determined using unpaired Student's *t*-test (two-tailed).

The finding that Fn14 is frequently overexpressed in mesenchymal subtype tumors has several clinical implications. First, Halliday *et al.*[101] recently reported that when mice bearing high-grade gliomas resembling the proneural GB subtype were exposed to ionizing radiation, there was a rapid change in the global gene expression pattern and these changes were indicative of a proneural to mesenchymal subtype phenotypic transition. This implies that Fn14 levels may increase following brain irradiation, which could in theory sensitize radioresistant GB cells to Fn14-targeted therapeutics (see below). Second, Sullivan *et al.*[102] reported in 2014 that GB patients contain circulating tumor cells, and that these cells express mesenchymal subtype classifier genes and have an invasive phenotype. Accordingly, if these circulating tumor cells express Fn14, one could potentially

use Fn14 mAbs as an affinity reagent as part of an immunoselection protocol to detect and capture this cell population (that is, liquid biopsy). The GB circulating tumor cells could then be subjected to genomic/transcriptomic analyses to guide patient therapy decisions.

Glioma cells that are migrating on ECM *in vitro* or invading into brain tissue *in vivo* express relatively high levels of Fn14 in comparison with stationary cells

Mariani *et al.*[103] reported that plating glioma cell lines on a glioma cell-derived ECM instead of an untreated or bovine serum albumin-treated plastic surface leads to an accelerated migratory rate, and cDNA microarray analysis identified *Fn14* as one of the genes that was most significantly upregulated in migration-activated cells. Elevated *Fn14* gene expression in migrating glioma cells was subsequently confirmed using northern and western blot analyses.[26] To test whether Fn14 was also overexpressed in glioma cells invading *in vivo*, Tran *et al.*[25] collected GB tumor core and invasive rim cells from three surgical specimens by laser capture microdissection, isolated RNA and examined Fn14 transcript levels by real-time quantitative PCR. They found a four- to sixfold induction of Fn14 transcript in the cells located at the rim compared with matched core cells. Two IHC studies have confirmed high *Fn14* gene expression in invasive rim cells.[25,104] In the most extensive study, Fortin *et al.*[104] prepared a glioma invasion tissue microarray containing representative punches of tumor core, edge and invasive rim from 44 GB specimens and Fn14 immunostaining intensity was scored as negative, weak, moderate or strong. They found that 94.4%, 88.4% and 100% of the core, edge and rim samples, respectively, had either moderate or strong Fn14 expression. A representative example of IHC staining of normal brain tissue and the invasive rim region of an Fn14-positive GB surgical specimen is shown in Figure 3. The molecular basis for elevated *Fn14* gene expression in migrating/invading glioma cells compared with stationary cells is not known; however, as mentioned above, *Fn14* is an NF-κB-inducible gene,[25,100] and Dhruv *et al.*[105] have reported that increased NF-κB activity is associated with glioma cell migration *in vitro* and invasion *in vivo*.

TWEAK treatment of Fn14-positive glioma cells stimulates migration, invasion and resistance to chemotherapeutic agents *in vitro*

Although TWEAK is the only known ligand for Fn14, there have been only three reports comparing *TWEAK* gene expression in normal brain versus brain tumors. Tran *et al.*[26] assayed TWEAK mRNA expression levels in normal brain versus GB tissue using real-time quantitative PCR and found that TWEAK mRNA was downregulated in 9/10 GB samples. In a second study by this same group, *TWEAK* gene expression levels were mined in an Affymetrix gene expression profile data set consisting of 24 non-neoplastic brain samples and 160 surgical samples representing different glioma tumor grades, including 82 GB specimens. TWEAK mRNA expression was detected but expression levels did not vary across the sample set.[25] In a recent report, TWEAK protein expression was detected in low- and high-grade glioma specimens by IHC.[97] Taken together, these studies indicate that the TWEAK cytokine is most likely present in the GB tissue microenvironment and for this reason it is of interest to determine the effect of TWEAK:Fn14 engagement on glioma cell biology.

It has been shown that TWEAK treatment of various Fn14-positive glioma cell lines (for example, SF767, U118, T98G, U87 and A172) stimulates cell migration in both a microliter-scale radial assay using laminin-coated glass slides[26,106,107] and in a modified Boyden chamber assay with TWEAK added to the lower well culture medium.[108] Important molecular mediators of this cellular response have been identified, including TNF receptor-associated factor-2,[107] the Src family kinase member Lyn,[108] the guanine nucleotide exchange factors SGEF, Ect2 and Trio, and the Rho GTPases Rac1, Cdc42 and RhoG.[106,107] Several of these signaling proteins, including Lyn[108] and SGEF,[107] are overexpressed in GB tumors. Recently, Cherry *et al.*[109] reported that TWEAK also stimulates glioma cell invasion using a three-dimensional type 1 collagen matrix assay. They also found that TWEAK:Fn14 engagement increased matrix metalloprotease-9 gene expression, consistent with a previous report using T98G glioma cells,[110] and that TWEAK pro-invasive activity was reduced in the presence of the pan-matrix metalloprotease inhibitor GM6001.

Finally, two studies have demonstrated that activation of the TWEAK/Fn14 axis can promote glioma cell survival following exposure to the pro-apoptotic TNF superfamily member TNF-related apoptosis-inducing ligand or the chemotherapeutic agents camptothecin (a DNA topoisomerase 1 inhibitor) and temozolomide (mentioned above).[104,111] TWEAK-triggered cell survival is mediated, at least in part, by activation of the NF-κB and Akt2 signaling pathways, and the enhanced expression of the anti-apoptotic proteins Bcl-X_L and Bcl-W.[104,111] Interestingly, IHC analysis of 44 GB specimens revealed a positive correlation between Fn14 and Akt2 expression levels.[104] A summary of the molecular pathways currently implicated in TWEAK regulation of

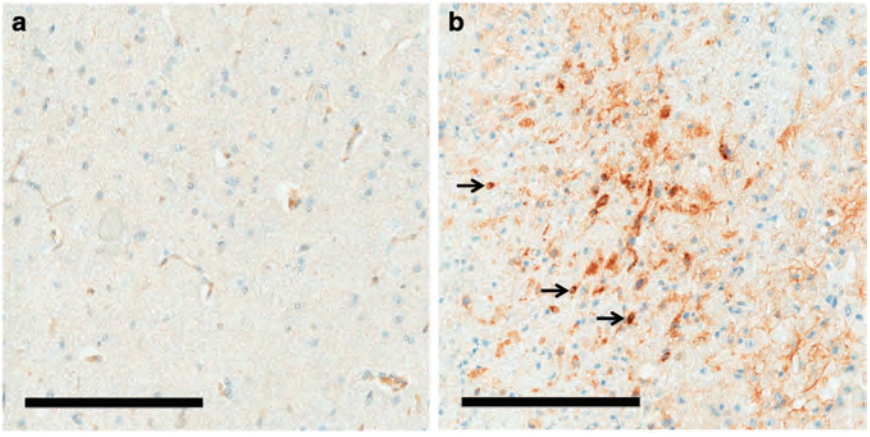

Figure 3. Analysis of Fn14 protein expression in normal brain and GB invasive rim tissue. Non-neoplastic brain tissue from a male 45-year-old epilepsy patient (**a**) and right frontal tumor tissue from a female 59-year-old GB patient (**b**) was immunostained using an anti-Fn14 rabbit mAb (EPR3179; Abcam, Cambridge, MA, USA). The GB image shows the invasive rim region, with the Fn14-positive glioma cells (brown stain, several positive cells are denoted with arrows) interspersed with normal brain tissue. Scale bar = 200 μm.

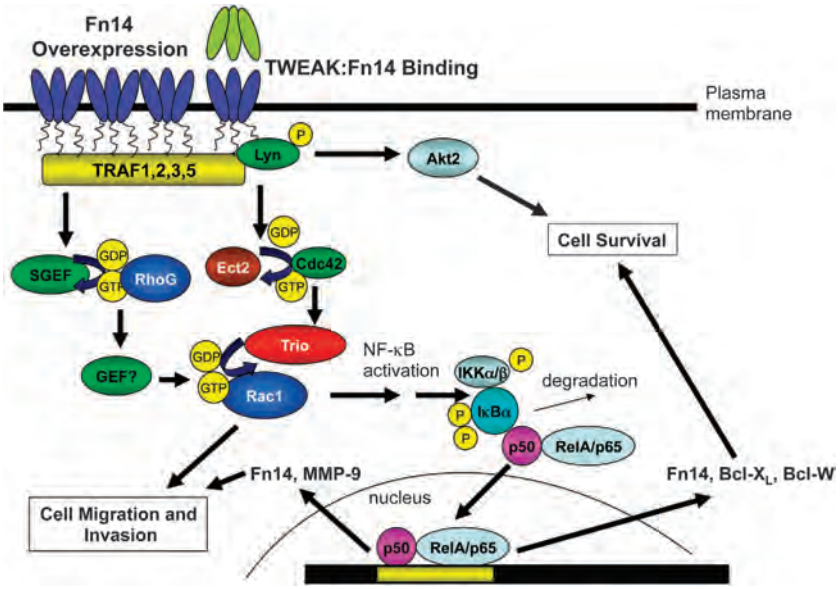

Figure 4. Summary diagram of signaling pathways and NF-κB-inducible gene products implicated in Fn14-triggered glioma cell migration, invasion and survival. TWEAK:Fn14 binding and Fn14 overexpression stimulate similar intracellular signaling cascades and downstream cellular responses. Some of the pathways and molecules that have a role in Fn14-mediated glioma cell migration, invasion and resistance to chemotherapeutic and pro-apoptotic agents are shown here.

glioma cell migration, invasion and survival is shown in Figure 4. Many of these same pathways may be stimulated by TWEAK-independent Fn14 activation as well (see below).

High Fn14 expression levels in glioma cells appears to trigger the same cellular responses that are observed after TWEAK treatment of Fn14-positive cells

Several studies have demonstrated that forced Fn14 overexpression in glioma cells can activate intracellular signaling[25,111] and promote migration,[25,110] invasion[25,106] and cell survival.[111] For example, Fortin *et al.*[106] reported that adenovirus-mediated overexpression of full-length, wild-type Fn14, but not an Fn14 cytoplasmic tail deletion mutant that is unable to bind TNF receptor-associated factors and activate intracellular signaling cascades, increases U118 and T98G glioma cell invasion in an *ex vivo* rat brain slice invasion assay. These authors also assessed the ability of Fn14 to promote mouse astrocyte migration *in vivo* using the replication-competent avian sarcoma leukosis virus long-terminal repeat with splice acceptor/tumor virus A gene delivery system.[112] For these experiments, chicken DF-1 cells infected with an replication-competent avian sarcoma-Fn14 construct were delivered intracranially into transgenic mice engineered to express tumor virus A, the avian cell surface receptor for the replication-competent avian sarcoma virus, under control of the astrocyte-specific glial fibrilic acid protein promoter. They found that forced Fn14 expression induced mouse astrocyte migration in the brain.

One issue with the Fn14 overexpression studies described above is the possibility that the amount of Fn14 produced in cells engineered to overexpress this receptor may greatly exceed the Fn14 levels found in glioma cells *in vivo,* which could trigger non-physiological Fn14 functions. Accordingly, it is important to note that Fn14 siRNA-mediated downregulation of endogenously expressed Fn14 in glioma cells reduces invasive capacity in the rat brain slice invasion assay.[25]

Taken together, these Fn14 overexpression/depletion studies support the possibility that elevated *Fn14* gene expression in GB tumors may have important biological consequences (for example, increase glioma cell invasiveness) even in the absence of the Fn14 ligand TWEAK. Indirect evidence supporting the concept of TWEAK-independent Fn14 signaling was summarized in two earlier review articles;[8,113] however, the strongest experimental evidence for this signaling mechanism was published in 2013.[114] In this report, Brown *et al.*[114] showed that the transient overexpression of an Fn14 mutant that is unable to bind TWEAK can activate the NF-κB pathway. The most likely explanation for this finding is that Fn14 overexpression in cells triggers receptor self-association ('multimerization'), which leads to TNF receptor-associated factor binding and downstream signaling. If Fn14 expression levels alone can regulate Fn14 signaling output *in vivo*, then this would have obvious clinical implications. Specifically, if TWEAK-independent Fn14 signaling is the main driver of GB pathophysiology, then TWEAK/Fn14 axis-directed therapeutic agents that act by blocking TWEAK binding to Fn14-positive cells may not be ideal for GB patient use. Several agents of this type have been described in the literature, including TWEAK-neutralizing mAbs,[22,115] Fn14-directed mAbs[116,117] and the Fn14-binding small molecule L524-0366.[118]

Therapeutic strategies that leverage Fn14 overexpression in GB tumors as a way to deliver cytotoxins or chemotherapy drugs to glioma cells while sparing healthy neurons and glial cells

Comprehensive overviews of the various TWEAK/Fn14 axis-targeted therapeutic agents in development were published recently.[35,36] Here we will focus on two therapeutic strategies that use Fn14 as a cell surface portal for the delivery of pro-apoptotic proteins or chemotherapeutic agents into cancer cells; in the context of GB, the ultimate goal is to kill the Fn14-positive invasive rim cells that cannot be safely removed by surgery. The proposal that Fn14 may be an excellent drug delivery portal is supported by recent work by Gurunathan *et al.*[119] demonstrating that the steady-state Fn14 expression level in most cells reflects a dynamic process, whereby Fn14 is constitutively synthesized, trafficked to the plasma membrane, internalized and degraded. In particular, constitutive Fn14 expression and endocytosis may result in more efficient and sustained delivery of Fn14-targeted therapeutics into the intracellular compartment.

Strategy 1: Fn14-targeted, fully humanized recombinant fusion proteins with cytotoxic activity

Targeted toxins are a class of cancer therapeutics that bind to cell surface proteins (usually plasma membrane-anchored receptors), become internalized via receptor-mediated endocytosis and release a cytotoxic payload.[120] The cell surface proteins targeted by these therapeutics are expressed at higher levels in tumors when compared with the corresponding normal tissue type; consequently, they are often described as tumor-associated antigens. Targeted toxins are typically recombinant fusion proteins consisting of a targeting moiety (for example, a receptor ligand or receptor-specific mAb fragment; mAb-based targeted toxins are generally referred to as 'immunotoxins') and a cell-killing moiety (for example, *Pseudomonas aeruginosa* exotoxin A or *Cornynebacterium diphtheriae* toxin). Targeted toxins that use modified bacterial, fungal or plant toxins as their cell-killing moiety all act on eukaryotic cells via inhibition of protein synthesis. A number of targeted toxins have been developed for potential use in GB patients and several have advanced to human clinical trials.[121,122] Some of the 'GB tumor-specific' cell surface proteins targeted by these constructs include podoplanin,[123] glycoprotein NMB,[124] ephrin type-A receptor 2,[125] urokinase plasminogen activator receptor,[126,127] transferrin receptor,[128] epidermal growth factor receptor (EGFR),[129,130] the constitutively activated EGFRvIII deletion mutant,[131] interleukin (IL)-4Rα,[132,133] and IL-13Rα2.[85,134,135] Bispecific constructs targeting two different GB cell surface proteins have also been described.[121,122,136] Only one targeted toxin, IL13-PE38QQR (Cintredekin Besudotox), a fusion protein composed of human IL-13 and a truncated, mutated version of *P. aeruginosa* exotoxin A, has been evaluated in a large phase III randomized GB clinical trial (the PRECISE trial).[137] In this trial, IL13-PE38QQR was administered via CED and efficacy was compared with Gliadel wafer implantation. Although the targeted toxin was well tolerated, there was no statistically significant improvement in survival compared with the wafer. Several possible reasons for this outcome have been proposed, including the likelihood that perhaps $<50\%$ of the patients enrolled in the trial may have actually harbored IL-13Rα2-positive tumors,[138] suboptimal catheter placement[85–88] and poor drug distribution.[88]

As Fn14 is frequently overexpressed in a wide variety of human primary cancers and metastases (discussed above), it is a cell surface target for precision therapeutic development for agents such as targeted toxins. In the context of GB therapy, Fn14 is perhaps a more appealing target than many of the other cell surface receptors mentioned above, because (i) there is minimal expression in normal brain and most GB tumors are Fn14-positive (~70–85%), (ii) Fn14 overexpression has been confirmed in both the stationary and invasive glioma cell populations and (iii) Fn14 undergoes constitutive receptor internalization, which could facilitate toxin delivery (discussed above). In addition, in the context of choosing a therapeutic strategy best suited for killing Fn14-positive invading glioma cells, it is of interest to note that there is evidence that glioma cell migration/invasion and proliferation are mutually exclusive processes, that is, migrating cells are not proliferating and vice versa (the 'Go or Grow' hypothesis).[105,139,140] Thus, Fn14-targeted toxins may be particularly useful for eradication of invasive glioma cells, because targeted toxins, unlike traditional chemotherapeutics or mAb-drug conjugates, can effectively kill quiescent, non-dividing cells.[120]

Rosenblum and colleagues[30,33,141,142] have developed several different Fn14-targeted toxins for cancer therapy. The first generation constructs consisted of either the anti-Fn14 mAb ITEM4 (IT4) or a humanized IT4 scFv fragment as the targeting moiety and the plant toxin gelonin as the therapeutic payload. These agents are internalized by Fn14-positive cancer cells, have cytotoxic activity when assayed on a large panel of cancer cell lines *in vitro* and inhibit xenograft tumor growth when delivered intravenously into immunodeficient mice.[33,141,142] The most recently described Fn14-targeted toxins use either human TWEAK or a humanized IT4 scFv fragment as the targeting moiety and the human pro-apoptotic serine protease granzyme B (GrB) as the cell-killing component.[30] These two fusion proteins, unlike all of the targeted toxins employed in the GB clinical trials described to date, are completely human constructs; accordingly, they are not likely to elicit immune responses in patients (which would prevent retreatment).[120] Both GrB-TWEAK and GrB-Fc-IT4 have been shown to display potent cytotoxic activity on Fn14-positive cancer cells and can inhibit the growth of established xenograft tumors in mice.[30] In regard to GB cells, GrB-TWEAK has been shown to promote A172 and U87 cell death *in vitro*,[30] but GrB-Fc-IT4 has not been tested and *in vivo* studies have not yet been conducted. As the therapeutic goal is to eradicate the invading glioma cells, GB patient-derived xenograft models are best suited for the *in vivo* efficacy experiments since patient-derived xenograft tumors generally demonstrate invasive intracranial growth in immunodeficient mice.[143,144] In addition, for these patient-derived xenograft experiments, it would be desirable to compare two delivery strategies: (i) local administration via new generation CED technology[84,145] and (ii) systemic administration in conjunction with one of the new approaches under development for localized BBB disruption, such as magnetic resonance-guided focused ultrasound.[77,146,147]

Strategy 2: Fn14-targeted, brain-penetrating therapeutic NPs

Nanotechnology and, in particular, the use of engineered nanocarriers to deliver imaging agents, cytotoxic drugs, RNA-based therapeutics or gene vectors to solid tumors, has the potential to revolutionize cancer diagnosis and therapy.[148–152] Many different nanoparticulate drug delivery systems have been developed for cancer therapy, including liposomes, polymeric NPs, micelles, dendrimers and polymer-drug conjugates.[148,150,153,154] In general, NP-based chemotherapeutic drug delivery has several advantages over conventional drug administration, including (i) preferential tumor accumulation, which is largely mediated by unique structural alterations in the tumor capillary and lymphatic vascular networks, resulting in a phenomenon known as the enhanced permeability and retention effect,[149,155–157] and (ii) sustained and, in some cases, controllable drug release at the tumor site.[148,149,154,158] These properties and others allow systemically administered nanotherapeutics to achieve higher intratumoral drug concentrations than their free drug counterparts, while simultaneously minimizing off-target toxicity in normal tissues, which can translate into improved therapeutic efficacy. One strategy that has been employed by numerous groups in order to further increase tumor cell-specific cytotoxicity *in vivo* is to decorate the surface of therapeutic nanocarriers with peptides, polypeptide ligands or mAbs recognizing molecules overexpressed on the cancer cell plasma membrane.[157,159,160] This approach is frequently referred to as 'active tumor targeting', to distinguish it from the 'passive tumor-targeting' mechanism that occurs via the enhanced permeability and retention effect.[157]

Several nanomedicines for non-central nervous system cancers have been approved by the Food and Drug Administration for clinical use, including Doxil (Janssen Biotech Inc., Horsham, PA, USA), a polyethylene glycol (PEG)-modified liposomal formulation of the anthracycline doxorubicin, and Abraxane (Celgene Corporation, Summit, NJ, USA), an albumin-bound NP formulation of the mitotic inhibitor paclitaxel.[149–152,154,158] These formulations are less toxic than their free drug counterparts but only modestly improve patient overall survival,[149,151] most likely owing to poor penetration through tumor tissue.[64,161] Indeed, it has been reported that the penetration depth of intravenously

administered Doxil in lung cancer xenograft tissue is only 8–16 μm (1–2 cell layers) beyond the tumor capillary endothelium.[162]

Numerous research groups are developing nanotechnology-based biomaterials that can be used for improved imaging and treatment of advanced brain cancers, including GB.[163–165] In regard to the design of therapeutic, biodegradable nanocarriers for GB patient use, the issue of poor nanomedicine tissue penetration discussed above is of particular concern, as perhaps the best strategy is to deliver the therapeutic particles into the invasive rim at the time of surgery in order to potentially reach and kill the unresected glioma cells embedded within the normal brain parenchyma (Figure 5a). As mentioned above, CED can 'infuse' therapeutic agents, including NPs,[93,166,167] into brain tissue but there is still limited drug distribution due in part to the brain penetration barrier; accordingly, several approaches have been pursued to develop nanocarriers with enhanced brain-penetrating capacity. These approaches include NP surface alterations such as (i) high-density PEGylation to minimize nonspecific binding to brain ECM,[168–170] and (ii) covalent conjugation of the tissue penetration peptide iRGD.[171–173] In addition, Zhou *et al.*[167] reported that relatively small (~70 nm) NPs exhibit enhanced ability to distribute in the brain following CED if they are stored in trehalose to reduce particle aggregation. There is also a significant amount of research underway evaluating whether nanomedicines engineered for 'active tumor targeting' may be the best formulations for GB patient therapy. Surface-modified NPs have been designed to recognize many of the same 'GB tumor-specific' cell surface proteins describe above for targeted toxin therapy, including transferrin receptor,[174,175] EGFR,[147] EGFRvIII,[176] IL-13Rα 2[177] and Fn14 (see below).

Schneider *et al.*[170] reported the successful engineering of polystyrene NPs with both brain-penetrating ability and an active tumor-targeting function. In this study, a dense low-molecular-weight PEG surface coating was employed in order to increase NP penetration into brain tissue and the NPs were targeted to Fn14-positive glioma cells by attaching the Fn14 mAb IT4 to the PEG chains. These Fn14-targeted, brain-penetrating NPs were able to (i) selectively bind to recombinant Fn14 but not brain ECM proteins in surface plasmon resonance (BiaCore) assays, (ii) exhibit increased association with Fn14-positive cells in comparison with Fn14-negative cells and (iii) rapidly diffuse within brain tissue, even though the PEGylated surface was decorated with mAb molecules. In addition, when administered intracranially, the Fn14-targeted, penetrating NPs showed improved tumor cell co-localization in mice bearing human GB xenografts compared with non-targeted, penetrating NPs. It is anticipated that NP formulations of this type might prove more successful that 'conventional' NPs in delivering chemotherapy drugs to the invading glioma cell population while sparing normal healthy brain cells (Figure 5b). Additional experimentation is required to determine whether the knowledge gained in this initial proof-of-concept study can be translated to the development of Fn14-targeted, drug-loaded, biodegradable penetrating NPs that can be tested for therapeutic efficacy in invasive patient-derived xenograft and transgenic rodent models of human GB.

CONCLUSIONS

GB is a lethal disease, in large part due to the highly invasive nature of malignant glioma cells, which limits complete surgical removal and dosing of adjuvant therapies owing to the involvement of functioning brain tissue. The proliferation of these cells within the normal brain parenchyma is the basis for tumor recurrence. Tran *et al.*[26] reported for the first time that the TWEAK receptor Fn14 was highly expressed in GB tissue specimens in comparison with non-neoplastic brain tissue. Since that time,

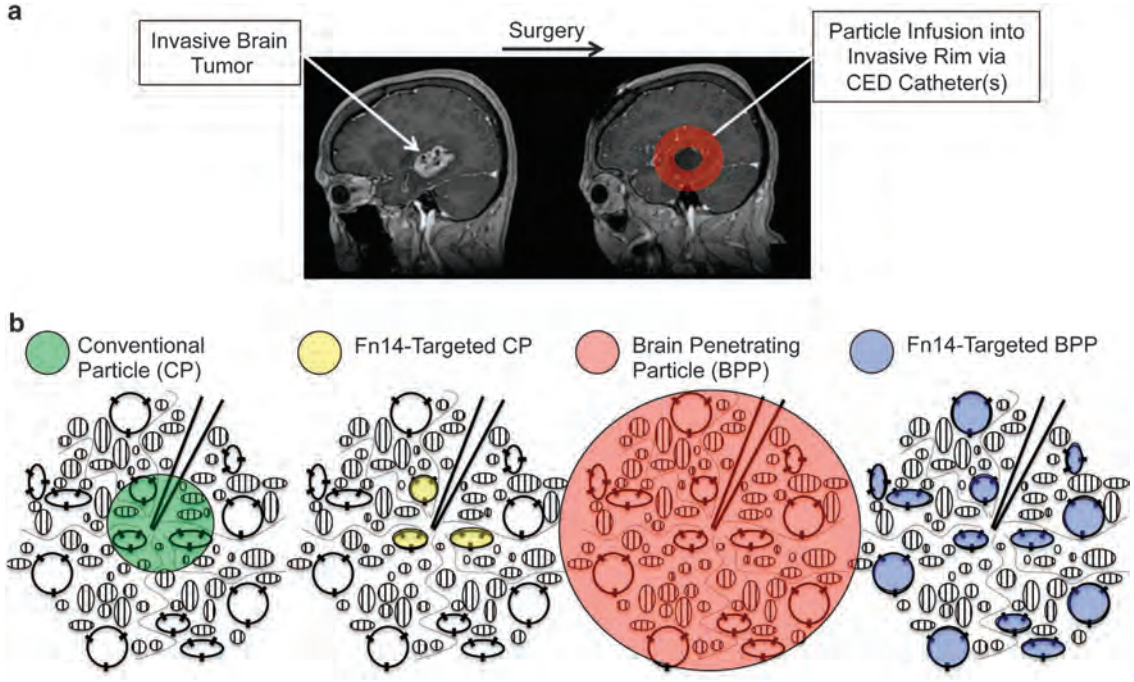

Figure 5. Adjuvant therapy for GB patients using engineered nanoparticles. (**a**) Magnetic resonance images obtained from a female 59-year-old GB patient before and after surgical resection are shown. Therapeutic nanoparticles delivered into the invasive rim region at the time of surgery using advanced CED technology could potentially reach the invasive glioma cells outside the immediate resection margin and deliver their cytotoxic payload. **(b)** Conventional, drug-loaded NPs without a specialized surface coating penetrate poorly through brain extracellular space after CED (normal brain cells indicated with white circles containing black lines; catheter is indicated using dark black V) and Fn14 targeting is restricted to those glioma cells close to the catheter entry point (white circles; Fn14 molecules indicated with rectangles). In contrast, NPs engineered for enhanced brain-penetrating capacity move deeper into the adjacent brain parenchyma and surface decoration with an Fn14 targeting moiety may increase NP binding to the invasive glioma cells.

numerous reports have confirmed and extended these results;[25,94–97] in addition, studies have revealed that Fn14 is overexpressed in invasive glioma cells *in vivo*[25,104] (Figure 3), and that manipulating Fn14 expression levels in glioma cells can have an impact on invasion capacity.[25,106] *Fn14* gene mutations have not yet been identified in GB tissue (COSMIC v72); therefore, Fn14 overexpression, which likely leads to constitutive Fn14 signaling,[114] may be the primary Fn14 alteration in this cancer type. Taken together, these studies have identified Fn14 as a promising potential cell surface target for GB therapeutics. The list of possible strategies that could be considered for the development of effective Fn14-targeted therapeutic agents for GB patients is somewhat limited, because Fn14 is not a protein kinase and Fn14-specific mAbs frequently exhibit agonist activity,[35,116,117] which could potentially promote glioma cell invasive activity. Here we have described two distinct therapeutic strategies in preclinical development that are designed to exploit glioma cell surface-specific Fn14 overexpression in order to deliver either humanized cytotoxins[30] or chemotherapeutic drug-loaded NPs[170] to malignant, invading glioma cells, while sparing healthy neurons and glial cells. Additional studies are necessary to elucidate whether these therapeutic strategies can be advanced beyond the preclinical stage and ultimately make a significant impact on GB patient survival.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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