Antitumor Activity of a Humanized, Bivalent Immunotoxin
Targeting Fn14-Positive Solid Tumors

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Abstract
The TNF-like weak inducer of apoptosis (TWEAK; TNFSF12) receptor Fn14 (TNFRSF12A) is expressed at low levels in normal tissues but frequently highly expressed in a wide range of tumor types such as lung, melanoma, and breast, and therefore it is a potentially unique therapeutic target for these diverse tumor types. We have generated a recombinant protein containing a humanized, dimeric single-chain anti-fibroblast growth factor-inducible 14-kDa protein (Fn14) antibody fused to recombinant gelonin toxin as a potential therapeutic agent (designated hSGZ). The hSGZ immunotoxin is a highly potent and selective agent that kills Fn14-positive (Fn14+) tumor cells in vitro. Treatment of cells expressing the MDR protein MDR1 (ABCB1B) showed no cross-resistance to hSGZ. Induced overexpression of Fn14 levels in MCF7 cells through HER2 (ERBB2) signaling translated to an improved therapeutic index of hSGZ treatment. In combination with trastuzumab, hSGZ showed an additive or synergistic cytotoxic effect on HER2+/Fn14+ breast cancer cell lines. Also, hSGZ treatment inhibited Erb3/Akt signaling in HER2-overexpressing breast cancer cells. Pharmacokinetic studies in mice revealed that hSGZ exhibited a biphasic clearance from plasma with a rapid initial clearance (t1/2 = 1.26 hours) followed by a 1.7-fold longer plasma half-life (t1/2 = 2.79 hours). At 24, 48, and 72 hours after injection, uptake of the hSGZ tumor stroma and vasculature (5, 22). The correlation between hormone, progesterone, and HER2. These breast tumors generally display a more aggressive clinical course exacerbated by the lack of effective targeted therapies (3). There is a significant need to identify new targets and treatment approaches to overcome or suppress the emergence of resistance. Thus, translational studies to develop novel targeted therapeutics remain a clinical imperative.

Fibroblast growth factor–inducible 14-kDa protein (Fn14) is the cell surface receptor for the cytokine TNF-like weak inducer of apoptosis (TWEAK; ref. 4). This receptor has been proposed as a novel target for cancer therapy because of its strong overexpression in many types of solid tumors and the intrinsic tumor cell killing capacity of the TWEAK-Fn14 pathway (5–7). TWEAK triggers multiple cellular responses, including proliferation, migration, differentiation, apoptosis, angiogenesis, and inflammation through its cognate receptor Fn14 (5, 6). TWEAK-dependent Fn14 signaling can exert either protumorigenic (8) or antitumorigenic (9, 10) effects depending on the particular microenvironmental conditions in vivo (6). TWEAK-independent Fn14 signaling may promote tumor cell invasion and metastasis (6).

The Fn14 receptor is expressed at relatively low levels in normal tissues but is dramatically elevated in a wide variety of human tumor types (11–21) and also can be expressed by tumor stroma and vasculature (5, 22). The correlation between

Introduction
Breast cancer remains the leading cause of cancer-related death in females worldwide (1). Despite the robust clinical efficacy of trastuzumab (Herceptin; Genentech) in HER2-positive (HER2+) breast cancer, primary and secondary resistance is frequently encountered (2). Inherent tumor heterogeneity and upregulation of alternative survival pathways in tumor cells frequently lead to emergence of resistance and treatment failure. Triple-negative breast cancer (TNBC) is a subtype of
increased Fn14 expression and higher tumor grade and/or poor prognosis has been documented in glioma (12, 13), breast cancer (14, 23), esophageal cancer (15, 16), prostate cancer (17), gastric cancer (18), and bladder cancer (19). Two types of Fn14-targeted agents have been tested in preclinical cancer studies—agonist antibodies (10, 11, 23, 24) and immunotoxins (21, 25). Our group showed that an immunoconjugate designated ITEM4-rGel composed of a murine monoclonal antibody (mAb) targeting the Fn14 receptor and the recombinant toxin gelonin was highly efficacious in inhibiting tumor growth in vivo (25). To develop an Fn14-targeted immunotoxin more suitable for clinical use, we generated a humanized, dimeric single-chain ITEM-4 construct fused to rGel (designated hSGZ; ref. 21). The hSGZ construct was shown to rapidly internalize and deliver the rGel payload to the cytosol of tumor cells, where it enzymatically blocks protein synthesis. We have previously shown that hSGZ binds to the extracellular domain of Fn14 with high affinity (Kd = 1.4 nmol/L) and induces necrosis in Fn14-positive (Fn14+) melanoma target cells (21). In addition, treatment of melanoma cells with the hSGZ construct upregulated cellular Fn14 expression and triggered cell signaling events similar to the Fn14 ligand TWEAK. Administration of hSGZ also showed excellent efficacy in a melanoma xenograft model (21).

In the current study, we examined the efficacy of the hSGZ construct against breast tumor cell lines and examined hSGZ in combination with trastuzumab on HER2+ and Fn14+ breast tumor cell lines. Some cell lines showed either an additive or a synergistic cytotoxic effect. In addition, we found that breast tumor cells resistant to chemotherapeutic agents were not significantly cross-resistant to hSGZ. Targeting Fn14 by hSGZ resulted in inhibition of the Erb3/Akt signaling pathway in HER2-overexpressing breast cancer cells. We further examined the in vitro and in vivo efficacy of hSGZ on breast cancer cells and the pharmacokinetics and biodistribution of hSGZ in mice. These findings support the proposal that Fn14 is a potential therapeutic target for both HER2+ and TNBC as well as other Fn14 overexpressing tumors such as melanoma and warrant the clinical investigation of hSGZ as a novel targeted agent for these cancer subtypes.

Materials and Methods

Cell lines and reagents

Human breast cancer cell lines MDA-MB-231, MCF-7, eB1, BT-474, and SKBR3 were maintained in RPMI-1640 medium. MCF7/HER2 cells were provided by Dr. Dihua Yu [MD Anderson Cancer Center (MDACC), Houston, TX]. The stable luciferase-expressing line MDA-MB-231/Luc was generated and grown as described previously (26). Fn14-deficient mouse embryonic fibroblasts (MEF 3.5~/-) were maintained in Dulbecco's Modified Eagle Medium. All media contained 10% FBS. The MDR [P-glycoprotein (P-gp)—overexpressing] human melanoma cell line MDA-MB-435/LCC6MDR1 was established as previously described (27). The human ovarian cancer cell line HeyA8 and its MDR equivalent HeyA8-MDR were maintained as previously described (28).

Cell lines (MCF-7, BT-474, SKBR3, and MDA-MB-231) were validated by short-tandem repeat (STR) DNA fingerprinting using the AmpF/STR Identifier Kit according to the manufacturer's instructions (Applied Biosystems). The STR profiles were compared with known American Type Culture Collection fingerprints to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://bioinformatics.istge.it/clima/; Nucleic Acids Research 37:D925-D932 PMCID: PMC2686526) and to the MDACC fingerprint database. The STR profiles matched known DNA fingerprints or were unique. No additional authentication was done for other transformed cell lines in this study.

The murine IgG2b/k mAb ITEM-4 directed against human and mouse Fn14 receptor (29) and the generation of immunoconjugate ITEM4-rGel have been described previously (25). hSGZ was expressed in the soluble fraction of Escherichia coli and purified to homogeneity after two chromatographic steps: cobalt affinity and ion exchange (21). The HER2-specific mAb trastuzumab was purchased from the MDACC pharmacy.

Lactate dehydrogenase release assay

Lactate dehydrogenase (LDH) was measured using the LDH Cytotoxicity Detection Kit from Clontech Laboratories, Inc. according to the manufacturer's instructions.

Internalization analysis

Immunofluorescence-based internalization studies were done on MDA-MB-231 and MEF 3.5~/- cells as described previously (25).

Flow cytometry analysis for Fn14 cell surface expression

Flow cytometry analysis of cells stained with ITEM-4 was conducted as described previously (25).

Western blot analysis

Western blot analyses were conducted according to standard procedures. Antibodies used include phosphorylated HER2, HER2, phosphorylated ErbB-3 (Tyr 1328), ErbB3, phosphorylated AKT1/2/3 (Ser 473), AKT 1/2/3, phosphorylated ERK (Tyr 204), ERK2, phosphorylated MEK1/2 (Ser 218/Ser 222), MEK-1, p27, ITEM-4, NF-κB p52/p100, β-actin (all from Santa Cruz Biotechnology), and Fn14 polyclonal antibody (Cell Signaling Technology).

Combination studies of hSGZ with trastuzumab

Cells were plated into 96-well plates. After 24 hours, the cells were treated with drug-containing medium. At the end of the indicated incubation period, growth inhibition was assessed by crystal violet staining. IC₅₀ doses of either hSGZ or 50 or 100 μg/mL of trastuzumab were used in the combination...
studies. To determine the effects of drug sequencing, cells were treated with three different sequences: sequence I—cells were pretreated with a trastuzumab for 6 hours, followed by coadministration with hSGZ for 72 hours; sequence II—cells were pretreated with hSGZ for 6 hours, followed by coadministration with trastuzumab for 72 hours; and sequence III—coexposure of hSGZ and trastuzumab to the cells for 72 hours.

**Pharmacokinetic study**

The hSGZ was labeled with the near-IR (NIR) fluorescence dye IRDye 800CW (LI-COR Biosciences) according to the manufacturer’s protocol. Female BALB/c mice (5–7 weeks old) were injected (i.v. via tail vein) with 80 μg of IRDye 800CW-labeled hSGZ (IR-hSGZ; ratio of dye/protein = 0.5). Three mice at each time point were sacrificed at 10, 20, 40, and 60 minutes and 2, 4, 8, 10, 12, and 24 hours after administration. Blood samples were removed from the chest cavity and fluorescent activity assayed using the In Vivo Imaging Systems (IVIS) 200 system (Xenogen Corp., filter sets: excitation/emission, 760/800 nm). The results from plasma determinations of drug concentration were analyzed by a least squares nonlinear regression using WinNonlin 5.0.1 software (Pharsight Corp.).

**Biodistribution study**

Fifteen Nu/Nu mice bearing MDA-MB-231/Luc tumors received i.v. injection of IRDye 800CW-labeled hSGZ (dye/protein = 0.5) through the tail vein at a dose of 80 μg per mouse. At 24, 48, or 72 hours after injection, the mice (n = 5) were euthanized and dissected. Blood and organ tissues, including heart, liver, spleen, kidney, lung, stomach, intestine, uterus, skin, muscle, bone, brain, and tumor, were removed from each mouse for quantitative optical imaging. The tissues were weighed, and fluorescent activity of each tissue was measured using the IVIS 100 system (Xenogen Corp.) using the indocyanine green (ICG) filter sets (excitation/emission, 710–760/810–875 nm).

**In vivo efficacy studies**

Experimental animals were carried out according to a protocol approved by the Association for Assessment and Accreditation of Laboratory Animal Care–approved Animal Care and Use Facility at MDACC. Female BALB/c nude mice (6–8 weeks old) were injected (hind flank) s.c. with MDA-MB-231/Luc cells (8 × 10^5 cells/mouse; suspended in 100 μL of PBS mixed with 100 μL BD Matrigel). Once tumors reached a mean volume of approximately 100 mm^3, animals were treated (i.v. via tail vein) with PBS, ITEM-4, or with immunotoxins. Animals were monitored and tumors were measured every 2 to 3 days. Data are presented as mean tumor volume (mm^3) ± SD. Survival was calculated using a predefined cutoff volume of 1,200 mm^3 as a surrogate for mortality (30). Average percentage weight change was used as a surrogate endpoint for tolerability. Toxicity was defined as 20% or more of mice showing 20% or more body weight loss and/or mortality.

**Bioluminescence imaging**

*In vivo* bioluminescence imaging (BLI) was conducted using the IVIS 100 system with Living Image acquisition and analysis software (Caliper Life Sciences). Anesthetized mice were intraperitoneally injected with 75 mg/kg p-luciferin and imaged 10 minutes after luciferin injection.

**Localization of hSGZ in tumor tissue**

Twenty-four hours after i.v. injection of ITEM-4, ITEM4-rGel, or hSGZ, the mice were sacrificed and tumor samples were collected and frozen immediately for sectioning. Localization of ITEM4-rGel and hSGZ in tumor tissues was conducted as previously described (21).

**Statistical analysis**

Statistical analyses were conducted using GraphPad Prism with one-way ANOVA followed by Dunnett test to compare the tumor sizes between the control- and drug-treated groups. Survival comparisons between groups were analyzed by log-rank test (GraphPad Prism 5). Differences between groups were considered significant when the P value was 0.05 or less.

**Results**

**Effect of hSGZ on breast tumor cell lines**

MDA-MB-231 is a triple-negative human breast cancer cell line that expresses relatively high levels of Fn14 (14). To determine whether hSGZ could specifically internalize into these cells, immunofluorescence staining was conducted on Fn14^+ and Fn14^- cells. Confocal microscopy images showed that the rGel moiety of hSGZ was observed primarily in the cytosol of Fn14-expressing MDA-MB-231 breast cancer cells (Fig. 1A) and MDA-MB-435 melanoma cells (Supplementary Fig. S1A). We found no internalization in Fn14-deficient MEF 3.5^-/- cells (data not shown).

Treatment of MDA-MB-231 cells with hSGZ has a comparative cytotoxicity to the parental immunonconjugate ITEM4-rGel with an IC50 of 0.1 nmol/L, and this effect can be completely abrogated by pretreatment with the mAb ITEM-4 (Fig. 1B). Exposure to hSGZ for as short a period as 1 hour, followed by drug removal and an additional 72-hour incubation period, resulted in no significant difference in cytotoxicity compared with 72-hour exposure (Fig. 1C; P > 0.05), indicating a rapid uptake mechanism. Additional cytotoxicity assays using a panel of breast cancer cell lines, which expressed different levels of Fn14, showed that hSGZ was highly toxic to Fn14^- cells (IC50 ranged from 0.1 to 2.4 nmol/L) and 188- to 8,350-fold more potent than free rGel. In contrast, hSGZ treatment had no significant effect on Fn14^- BT-474 and BT-474/HR (Herceptin-resistant) breast cancer cells (Table 1) or Fn14-deficient MEF 3.5^-/- cells (Supplementary Fig. S1B). We reported previously that hSGZ-mediated cytotoxicity in melanoma cells occurred via a necrotic mechanism (21). Treatment of MDA-MB-231 cells for 72 hours did not induce apoptosis (Supplementary Fig. S1C). To assess whether necrotic cell death was induced in breast cancer cells, an LDH release assay was used (31). As shown in Fig. 1D, treatment with hSGZ induced LDH release, which was found to be both time- and dose-dependent and which is consistent with a necrotic cell death mechanism.
MDR1 tumor cells are not cross-resistant to ITEM4-rGel or hSGZ

Expression of P-gp encoded by the mdr1 gene is one of the key molecules leading to resistance of cancer cells to chemotherapeutic agents (32). To evaluate the effect of MDR1 expression on immunotoxin-induced cell killing, we compared the sensitivities of MDA-MB-435/LCC6MDR1 and HeyA8-MDR cells and their parental counterparts (MDA-MB-435 and HeyA8) to Fn14-targeted immunotoxins. We also tested the activity of two other cytotoxic compounds—paclitaxel and doxorubicin, which have previously been shown to be MDR1 substrates (32, 33). As expected, paclitaxel and doxorubicin were less potent in killing the P-gp-overexpressing human melanoma MDA-MB-435/LCC6MDR1 cells and the paclitaxel-derived ovarian cancer HeyA8-MDR cells than in killing their corresponding parental cells (Table 2). These results indicate that MDR1 activity limits the potency of these conventional cytotoxic compounds. In contrast, the IC50 of ITEM4-rGel and hSGZ on the MDR cells were similar to their parental cells (less than 1-fold for both MDR cell lines, respectively; Table 2), suggesting that rGel-based immunotoxins may be effective in overcoming MDR1-mediated MDR in cancer.

Figure 1. Effect of the humanized, dimeric single-chain immunotoxin hSGZ on MDA-MB-231 cells. A, cells were either left untreated or treated with 50 nmol/L rGel and 50 or 100 nmol/L hSGZ for 4 hours. The cells were fixed, acid washed to remove surface-bound material, permeabilized, and immunostained for the presence of rGel (green). The cells were counterstained with propidium iodide (red) to identify nuclei and visualized using a confocal microscope. Bar, 50 μm. B, cells were treated with indicated concentration of rGel, ITEM4-rGel, or hSGZ for 72 hours. In addition, cells were pretreated with 1 μmol/L ITEM-4 for 2 hours and then coincubated with a different concentration of hSGZ for another 72 hours. Cell viability was assessed by crystal violet staining. C, cells were plated and then exposed to hSGZ at 0.3 nmol/L for indicated periods of time. Cell viability was assessed at 72 hours as described previously. D, cells were treated with different concentrations of hSGZ for 24 or 48 hours, and LDH release was measured. Treatment of cells with Triton X-100 served as a positive control causing maximum LDH release. Results represent mean ± SD, n = 3.
hSGZ inhibits Erb3/Akt signaling in HER2-overexpressing breast cancer cells

Fn14 and HER2 are frequently coexpressed in human breast tumors (14, 23), and HER2 signaling in breast cancer cells directly induces Fn14 gene expression (34). To determine if HER2-induced upregulation of Fn14 expression would sensitize cells to hSGZ treatment, MCF7 and MCF7/HER2 cells were exposed to hSGZ. Increased Fn14 protein expression by HER2 overexpression was confirmed by flow cytometry (Fig. 2A). MCF7/HER2 cells were more sensitive to hSGZ treatment with a 3.4-fold decreased IC50 dose compared with MCF7 cells (Fig. 2B). This finding suggests that induced overexpression of Fn14 by HER2 signaling could be translated to an improved therapeutic index with immunotoxin treatment.

The two most prevalent downstream signaling pathways activated in HER2-overexpressing breast cancer cells are the Ras/mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K)/AKT pathways. To further understand the mechanism of action and potency of hSGZ on HER2+ breast cancer cells, we explored the HER2 signaling pathway in MCF7 and MCF7/HER2 cells in the absence or presence of hSGZ. As shown in Fig. 2C, HER2 overexpression induced robust phosphorylation of HER2, HER3 (ErbB3), and Akt in MCF7 cells as assessed by Western blot analysis. In MCF7/HER2 cells, hSGZ significantly suppressed HER3 and Akt phosphorylation in a dose-dependent manner without affecting HER2 phosphorylation (Fig. 2C), hSGZ treatment also decreased the total level of HER3 in MCF7 cells but not in MCF7/HER2 cells. Overexpression of HER2 in MCF7 cells increased Fn14 expression as expected (34); however, treatment with hSGZ also dose-dependently increased Fn14 levels (Fig. 2C), which is consistent with our previous report showing that hSGZ can upregulate Fn14 expression (21). hSGZ treatment dose-dependently suppressed Erk2 and phospho-Erk1/2 levels (Fig. 2D). Treatment also increased both NF-kB p52 and NF-kB p100 levels in both cell lines and decreased p27 levels (Fig. 2D). This latter effect is probably due to a decrease in regulatory, rapid-turnover proteins as a result of the overall inhibition of protein synthesis by the rGel component of the immunotoxin. These results indicate that the Fn14 receptor may be an important downstream regulator of HER2/HER3 signaling in breast cancer cells and that hSGZ treatment can suppress HER3/Akt activation in HER2-overexpressing cells.

hSGZ in combination with trastuzumab shows additive/synergistic effects on HER2+/Fn14+ breast cancer cells

We next examined the effects of hSGZ in combination with trastuzumab, which is currently used to treat patients with

### Table 1. Cytotoxicity of hSGZ on breast cancer cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Fn14 receptor</th>
<th>HER2 receptor</th>
<th>rGel IC50 (nmol/L)</th>
<th>hSGZ IC50 (nmol/L)</th>
<th>Targeting index</th>
</tr>
</thead>
<tbody>
<tr>
<td>eB1</td>
<td>++</td>
<td>++</td>
<td>835</td>
<td>0.1</td>
<td>8,350</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>+++</td>
<td>/C0/C0</td>
<td>745</td>
<td>0.1</td>
<td>7,450</td>
</tr>
<tr>
<td>SKBr-3</td>
<td>++</td>
<td>+++</td>
<td>1,550</td>
<td>0.6</td>
<td>2,538</td>
</tr>
<tr>
<td>MCF7/HER2</td>
<td>+++</td>
<td>/C0/C0</td>
<td>441</td>
<td>0.7  P&lt;0.05 (MCF7/HER2 vs. MCF7)</td>
<td>630</td>
</tr>
<tr>
<td>MCF7</td>
<td>+</td>
<td>--</td>
<td>452</td>
<td>2.4</td>
<td>188</td>
</tr>
<tr>
<td>BT-474/HR</td>
<td>--</td>
<td>+++</td>
<td>590</td>
<td>489 n.s. (BT-474/HR vs. BT-474)</td>
<td>1</td>
</tr>
<tr>
<td>BT-474</td>
<td>--</td>
<td>+++</td>
<td>945</td>
<td>&gt;489</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

Abbreviation: n.s., not statistically significant (P > 0.05).

Targeting index represents IC50 of rGel/IC50 of hSGZ. Student t test was used to calculate P value. Data are representative of three independent experiments.

### Table 2. In vitro cytotoxicity of paclitaxel, doxorubicin, ITEM4-rGel, hSGZ, and rGel for HeyA8-MDR, HeyA8, MDA-MB-435/LCC6MDR1, and MDA-MB-435 cells

<table>
<thead>
<tr>
<th>Cytotoxic agents</th>
<th>HeyA8-MDR IC50 (nmol/L)</th>
<th>HeyA8 IC50 (nmol/L)</th>
<th>Resistance fold</th>
<th>MB-435MDR1 IC50 (nmol/L)</th>
<th>MB-435 IC50 (nmol/L)</th>
<th>Resistance fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>70 ± 4</td>
<td>0.07 ± 0.02</td>
<td>1,000</td>
<td>36.5 ± 13.4</td>
<td>0.006 ± 0.001</td>
<td>6,083</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>151 ± 5</td>
<td>3.6 ± 3.1</td>
<td>42</td>
<td>796 ± 31.3</td>
<td>45.4 ± 29.4</td>
<td>17</td>
</tr>
<tr>
<td>ITEM4-rGel</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.007</td>
<td>1</td>
<td>0.003 ± 0.003</td>
<td>0.007 ± 0.004</td>
<td>0.5</td>
</tr>
<tr>
<td>hSGZ</td>
<td>1.8 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>1</td>
<td>0.008 ± 0.002</td>
<td>0.009 ± 0.007</td>
<td>0.9</td>
</tr>
<tr>
<td>rGel</td>
<td>140 ± 50</td>
<td>68 ± 25</td>
<td>2</td>
<td>282 ± 26</td>
<td>270 ± 27</td>
<td>1</td>
</tr>
</tbody>
</table>

NOTE: The data are presented as the mean IC50 ± SD from two independent experiments.

Resistance fold is defined as IC50 of drugs on MDR cells/IC50 of drugs on matched parental cells.
HER2-overexpressing breast cancer (35, 36), on MCF7 and MCF7/HER2 cells. As expected, treatment of HER2-negative (HER2*/C0) MCF7 cells with 50 or 100 μg/mL of trastuzumab had little effect on cell viability, and cotreatment with an IC25 dose of hSGZ produced a cytotoxic effect similar to that of hSGZ alone (Fig. 3A). In contrast, although 50 or 100 μg/mL of trastuzumab had a marginal growth inhibition effect on MCF7/HER2 cells, an IC25 dose of hSGZ, when combined with 50 or 100 μg/mL of trastuzumab, markedly increased its cytotoxic effect to approximately 50% of viability (Fig. 3A). hSGZ also potentiated the cytotoxic effect of trastuzumab on SKBR3 breast cancer cells, which have high endogenous expression levels of both the HER2 and Fn14 receptors (Fig. 3B). The data (Fig. 3B) suggest that simultaneous addition of hSGZ and trastuzumab was significantly (P < 0.05) more active than either addition of hSGZ followed by trastuzumab, or trastuzumab followed by hSGZ.

**Pharmacokinetics of hSGZ**

Pharmacokinetic data were obtained using hSGZ labeled with the NIR dye 800CW (IR-hSGZ; Supplementary Fig. S3A) after i.v. administration in mice. As shown in Fig. 4A, there was a biexponential disposition of IR-hSGZ in the blood. Following i.v. administration, IR-hSGZ had a rapid initial distribution to a highly perfused, central compartment (mean t1/2a = 1.26 hours), followed by an extensive distribution to a second tissue compartment (mean t1/2 b = 7.29 hours), during which the fluorescent-conjugate spread from the central blood compartment to the extravascular space of the solid tissues. The initial concentration of IR-hSGZ in the plasma was 24.9 ± 2.9 μg/mL, which corresponded to the injected dose of the drug. The pharmacokinetic parameters are summarized in Fig. 4A, right.

**Biodistribution of hSGZ**

NIR images were taken at 24, 48, and 72 hours postinjection of fluorescent-labeled hSGZ into nude mice bearing MDA-MB-231/Luc tumors and showed an accumulation of fluorescence in the organs and tumors (Fig. 4B and Supplementary Fig. S3B). BLI imaging of the MDA-MB-231/Luc primary tumors corresponding to the NIR images of the same mice clearly showed that the IR-hSGZ was colocalized in the tumor tissues (Fig. 4B). To avoid any measuring errors caused by limited tissue penetration of fluorophores, animals were sacrificed and tumor and major organs were collected at the 24-, 48-, and 72-hour time points and then subjected immediately to NIR imaging (Supplementary Fig. S3C). The injected dose per gram (%ID/g) tissue of IR-hSGZ in organs and tissues dissected at different
times after fluorescent tracer injection is summarized in Fig. 4C. The liver showed the highest uptake among the tissues studied (32.5% ± 3.9%, 27.7% ± 16.1%, and 9.2% ± 1.9% for 24, 48, and 72 hours, respectively). All the tissues except the tumors showed decreased drug uptake over time. However, the tumors showed the middle level of uptake among the tissues but with a steady pattern over time (5.1% ± 0.5%, 4.8% ± 1.1%, and 4.7% ± 1.4% for 24, 48, and 72 hours, respectively). Similar biodistribution patterns were observed when the data were plotted as a tissue-to-muscle ratio (Supplementary Fig. S3D). IR-hSGZ accumulation in the tumor was further indicated by the increasing tumor-to-muscle ratio post-IR-hSGZ injection over time (Supplementary Fig. S3D; 5.6 ± 1.3, 6.2 ± 1.6, and 9.0 ± 2.3 for 24, 48, and 72 hours, respectively).

Efficacy of hSGZ in breast cancer xenograft model

We next examined the effect of hSGZ on the growth of established tumors using a MDA-MB-231/Luc xenograft model of human breast cancer. Primary tumor sizes were assessed by either caliper measurement or BLI imaging. A significant tumor growth inhibition was observed at both doses of the hSGZ (25 or 36 mg/kg) as compared with saline control (P < 0.01; Fig. 5A). The tumors remained static for the entire study period of more than 40 days in response to a dose of 36 mg/kg for ITEM4-rGel (P = 0.009; Fig. 5A). Mice treated with 36 mg/kg of ITEM-4 plus rGel also had marginal tumor growth relative to the saline control, but there was no significant difference between these two groups (P = 0.06; on day 28). Survival in the mice treated with ITEM4-rGel and hSGZ was significantly better than in those treated with saline or with ITEM-4 plus rGel (P < 0.0001; Fig. 5B). Compared with animals in the saline group, ITEM-4 plus rGel–treated mice showed a 48% increased life span. The representative BLI images of the tumors on selected days clearly showed the antitumor properties of the ITEM4-rGel and hSGZ (Fig. 5C). Because the ITEM-4 antibody can recognize murine Fn14, we were also able to assess...
immunotoxin toxicity against normal mouse tissues. Toxicity was monitored by frequent body weight measurements. The mouse body weights showed less than a 20% change in any of the treated or control groups over the duration of the experiment (Fig. 5D). We also found Fn14 was upregulated after treatment with anti-Fn14 antibody ITEM-4 and ITEM4-based immunotoxins (ITEM4-rGel and hSGZ) in vivo as assayed by Western blot analysis of tumor tissues (Fig. 5E). Immunofluorescence staining confirmed that ITEM4-rGel and hSGZ localized specifically in tumor tissue, and no nonspecific staining was observed in tumors after administration of ITEM-4 plus rGel and saline detected by anti-rGel antibody (Supplementary Fig. S4).

Discussion

Three separate groups have now independently reported that Fn14 is frequently overexpressed in breast tumors compared with normal breast epithelium (11, 14, 22, 23). Overexpression of Fn14 was found to be positively correlated with metastasis, positive lymph nodes, and HER2+/ER/C0 status, which are three indicators of poor prognosis of patients with breast cancer (14, 23). Our group recently showed that Fn14 was overexpressed in 173 of 190 (92%) of melanoma biopsy specimens tested (21), suggesting that an anti-Fn14 approach could also be useful for this tumor type. The differential expression of Fn14 in HER2þ breast tumors compared with normal tissue prompted us to test the Fn14-targeted immunotoxin hSGZ as an alternative antitumor strategy beyond the currently used HER2-directed therapeutics trastuzumab and lapatinib. Because the cytotoxic mechanism of hSGZ is markedly distinct from that of these two agents, it is a potential alternative for patients with HER2þ breast cancer resistant to current therapeutic approaches.

The development of resistance to chemotherapeutic agents through emergence of tumor cells expressing a MDR phenotype (33) is a well-known and well-characterized event. The P-gp transporter (MDR1)-mediated efflux of...
anticancer drugs is the most commonly observed MDR phenotype clinically, and correlations between the transporter expression and poor response to chemotherapy have been documented for many cancer types (32, 37). The results of the cytotoxicity studies indicate that the ITEM4-rGel and hSGZ constructs display similar sensitivity to MDR-1–overexpressing tumor cell lines (Table 2), supporting the possibility that rGel-based immunotoxins may be effective in targeting solid tumors resistant to conventional agents.

Figure 5. Both ITEM4-rGel and hSGZ inhibit tumor growth and prolong survival in a MDA-MB-231/luc breast tumor xenograft model. MDA-MB-231/luc cells were implanted s.c. and groups of mice (n = 5) were treated (i.v. via tail vein) with saline, ITEM-4 plus rGel, ITEM4-rGel (36 mg/kg), and hSGZ (36 and 25 mg/kg) every 6 days starting when the tumors were approximately 100 mm³. Arrow indicates dosing days. A, efficacy data are plotted as mean tumor volume (in mm³)/C6 SEM. Tumor size was assessed by direct caliper measurement. B, survival data are plotted as percentage of animals surviving in each group using a predefined cutoff volume of 1,200 mm³ as a surrogate for survival. C, BLI images of mice on selected days are shown. D, percentage change in body weight of each group of mice is plotted as a function of time. E, tumor tissues from the xenograft experiment in A were analyzed for Fn14 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression by Western blot analysis.
The members of the HER family (ErbB1/EGFR, ErbB2/HER2, ErbB3, and ErbB4) of transmembrane proteins have been firmly established as important drivers and therapeutic targets in solid tumors (38, 39). Recently, Whitsett and colleagues (40) reported that high Fn14 levels correlated with EGFR (HER1) activation in non–small cell lung cancer specimens and cell lines. In addition, the Fn14 and HER2 receptors are frequently coexpressed in breast tumors (14, 23), and Fn14 is a HER2-inducible gene (34). We report here that the increased Fn14 expression by HER2 signaling in MCF-7 cells could be translated to a better therapeutic index by targeting Fn14 when using hSGZ (Fig. 2B). The correlation of Fn14 and ErbB family member activation suggests that therapeutic targeting of Fn14 in tumors driven by oncogenic ErbB family members could benefit patients.

HER2/ErbB3 heterodimers have been found to be powerful oncogene drivers, in part, because ErbB3 activation amplifies signaling through PI3K (41). It is thought that ErbB3 functions primarily to drive HER2-mediated PI3K signaling (42, 43). ErbB3 promotes HER2-induced changes in the breast epithelium before, during, and after tumor formation (44). Recent studies suggest that HER2-amplified breast cancer cells use HER3/ErbB3 to promote therapeutic resistance to HER2 inhibitors trastuzumab and the dual EGFR/ErbB2 tyrosine-kinase inhibitor lapatinib (45).

Because ErbB2 inhibitors initially cause a decrease in P-ErbB3/PI3K signaling (46–48), many metastatic HER2-amplified breast cancers do not respond or eventually escape trastuzumab- and lapatinib-mediated growth inhibition, often with recovery of ErbB3/PI3K signaling (45). Thus, the HER3-PI3K node is emerging as a potential target for anticancer therapy (49, 50). Our study shows that targeting Fn14 by hSGZ inhibits ErbB3/Akt signaling in HER2-overexpressing MCF-7 breast cancer cells (Fig. 2C). These results suggest that hSGZ has the potential to overcome trastuzumab or lapatinib resistance in HER2+ breast tumors.

Clinical trials using pertuzumab and trastuzumab in combination with advanced HER2+ breast cancers have shown significant clinical activity (51). Thus, a rationale exists for combining HER2-targeted agents with Fn14-targeted agents such as hSGZ. Our studies on MCF/HER2 cells clearly show that treatment with hSGZ augments the therapeutic efficacy of trastuzumab (Fig. 3). Furthermore, in vitro combination studies on MDA-MB-435 melanoma cells using hSGZ and several standard chemotherapeutic agents also showed a synergistic and/or additive cytotoxic effect (Supplementary Fig. S2). These data suggest that consideration should be given to eventual clinical trials with combinations of hSGZ and trastuzumab or standard chemotherapeutic agents.

The pharmacokinetic data for IR-hSGZ were best characterized by biexponential kinetics with a rapid initial clearance phase followed by a more prolonged clearance phase after i.v. administration (Fig. 4A). The prolonged disposition of IR-hSGZ in mice is likely attributable to the relatively large size of the dimerized fusion protein (120 kDa). The prolonged circulation of IR-hSGZ in blood allows for accumulation and retention of the fusion protein in the tumor over time, as shown in the NIR and BLI imaging study in mice bearing MDA-MB-231/Luc tumors (Fig. 4B and Supplementary Fig. S3). The tumor/muscle ratio of hSGZ was shown to increase over time and reached the highest level 72 hours after injection of the IR-hSGZ (Fig. 4C and Supplementary Fig. S3D), which suggests that hSGZ was efficiently delivered to the tumor site.

The biodistribution data obtained in our study should be useful for determining dosing schedules, establishing efficacy, and predicting possible toxicity. As expected, the highest levels of drug were found in highly perfused organs such as liver, kidneys, and spleen (Fig. 4C). Retention in the liver may also suggest that the liver may be involved in hSGZ metabolism, but no overt toxicity was observed in mice even at the highest doses used. It is important to note that the anti-Fn14 antibody used for hSGZ construction cross-reacts with murine Fn14 receptors. Therefore, any toxicity observed is likely to be more relevant to eventual clinical studies.

Our preclinical antitumor studies using a 5-day interval treatment schedule based on pharmacokinetic and imaging data showed therapeutic efficacy of hSGZ in MDA-MB-231/Luc tumors (Fig. 5). Consistent with our previous melanoma studies, we showed that ITEM4-rGel was more potent than hSGZ in this breast cancer xenograft model. However, hSGZ showed reduced systemic toxicity compared with ITEM4-rGel as assessed by body weight measurements (Fig. 5D). Fn14 expression was upregulated by treatment with hSGZ in vitro (Fig. 2C) and in vivo (Fig. 5E), which was consistent with our previous results (21).

Recently, we reported phase I clinical studies of an rGel immunoconjugate containing the anti-CD33 antibody HuM195. These studies showed limited antigenicity and no vascular leak issues with rGel-based therapeutics even after repeated administration (52). These promising preclinical activities of hSGZ will need to be confirmed by clinical trials. Overall, our data suggest that hSGZ is a potent anticancer agent with a novel mechanism of action that might prove to be effective against multiple Fn14+ tumor types resistant to conventional agents.

Disclosure of Potential Conflicts of Interest
J.A. Winkles is a consultant for arGEN-X. M.G. Rosenblum has ownership interest (including patents). No potential conflicts of interest were disclosed by the other authors.

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