Development and Characterization of a Potent Immunoconjugate Targeting the Fn14 Receptor on Solid Tumor Cells

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Running Title: Immunoconjugate Targeting Fn14 Receptor

Keywords: TWEAK, Fn14, immunoconjugate, ITEM4-rGel

Abbreviation list: TNF, Tumor necrosis factor; TWEAK, TNF-like weak inducer of apoptosis; Fn14, fibroblast growth factor -inducible immediate-early response protein 14; rGel, recombinant gelonin; HMGB1, the high-mobility group box 1 protein.

Notes:
Research conducted, in part, by the Clayton Foundation for Research (MGR), and supported by NIH grant NS055126 (JAW) and DOD Breast Cancer Concept Award BC086135 (JAW).

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Conflict of interest: None

Word count: 4,746  (Abstract to Acknowledgement section); 6 figures; 2 tables.
Abstract

TNF-like weak inducer of apoptosis (TWEAK) and FGF-inducible 14 (Fn14) are a TNF superfamily ligand-receptor pair involved in many cellular processes including proliferation, migration, differentiation, inflammation and angiogenesis. The Fn14 receptor is expressed at relatively low levels in normal tissues, but it is known to be dramatically elevated in a number of tumor types, including brain and breast tumors. Thus, it appears to be an excellent candidate for therapeutic intervention. We first analyzed Fn14 expression in human tumor cell lines. Fn14 was expressed in a variety of lines including breast, brain, bladder, skin, lung, ovarian, pancreatic, colon, prostate and cervical cancer cell lines. We then developed an immunoconjugate containing a high-affinity anti-Fn14 monoclonal antibody (ITEM-4) conjugated to recombinant gelonin (rGel), a highly cytotoxic, ribosome-inactivating n-glycosidase. Both ITEM-4 and the conjugate were found to bind to cells to an equivalent extent. Confocal microscopic analysis showed that ITEM4-rGel specifically and rapidly (within 2 h) internalized into Fn14-positive T-24 bladder cancer cells but not into Fn14-deficient mouse embryonic fibroblasts. Cytotoxicity studies against 22 different tumor cell lines showed that ITEM4-rGel was highly cytotoxic to Fn14-expressing cells and was 8 to 8 x10^4 fold more potent than free rGel. ITEM4-rGel was found to kill cells by inducing apoptosis with high-mobility group box 1 protein (HMGB1) release. ITEM4-rGel immunoconjugate administration promoted long-term tumor growth suppression in nude mice bearing T-24 human bladder cancer cell xenografts. Our data support the use of an antibody-drug conjugate approach to selectively target and inhibit the growth of Fn14-expressing tumors.
Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and fibroblast growth factor-inducible 14 (Fn14) are a TNF superfamily ligand-receptor pair implicated in the pathogenesis of several diseases including chronic inflammatory diseases, stroke and cancer (1). In the cancer setting, TWEAK and Fn14 are expressed in tumor tissue and TWEAK can activate several cellular processes associated with tumor progression such as proliferation, invasion, angiogenesis and inflammation (1, 2). However, the downstream functional consequences of TWEAK binding to Fn14 appear to depend on cellular context. For example, although TWEAK treatment of certain tumor cell lines can induce cell death (3, 4), TWEAK treatment of other tumor lines, for example glioma cell lines, does not cause cell death, but instead promotes cell migration (5) and enhanced survival following chemotherapeutic drug exposure (6, 7).

The Fn14 receptor is expressed at relatively low levels in normal tissues, but it is dramatically elevated locally in injured tissues, where it plays a role in tissue remodeling (1). In addition, the Fn14 gene is overexpressed in multiple solid tumor types relative to matched adjacent normal tissue or normal tissue from non-diseased donors (5, 8-12). Some of these prior Fn14 overexpression reports also included data indicating that Fn14 expression levels positively correlate with tumor progression (5, 10, 11) and poor patient outcome (9). The fact that Fn14 expression is elevated in tumors as compared to normal tissue suggests that it may be a potential tumor antigen, and therefore, on the basis of expression alone, a valuable therapeutic target. Recently, Culp et al. (8) reported that an anti-Fn14 monoclonal antibody (mAb) capable of inducing tumor cell apoptosis in vitro
was efficacious in a range of tumor xenograft models, including colorectal, breast, renal, skin and head/neck cancer models. These authors suggested that the antitumor effects occurred through both direct cell growth inhibition and antibody-dependent cellular cytotoxicity (ADCC) mechanisms. In consideration of these findings, this group and others (13) have proposed that therapeutic activation of the TWEAK/Fn14 pathway may represent a novel modality to inhibit tumor growth.

The use of monoclonal antibodies, ligands, DARPinns (designed ankyrin repeat proteins) (14), and adnectins (15) for the delivery of highly cytotoxic molecules to specific target cells has gained wide acceptance and significant prominence in the field of targeted therapy. There are now several antibody-drug conjugates in clinical development and there are a number of toxin-based therapeutics under development and approved for use (16, 17). The broad tumor expression coupled with limited normal expression makes Fn14 an attractive candidate for a targeted therapeutic approach. We have developed an immunoconjugate designated ITEM4-rGel containing a high-affinity anti-Fn14 monoclonal antibody conjugated to recombinant gelonin (rGel), a highly cytotoxic, ribosome-inactivating n-glycosidase. Herein, we report that this immunoconjugate can kill Fn14-positive tumor cells in vitro and inhibit tumor growth in vivo.
Materials and Methods

Materials

Recombinant gelonin was obtained from Goodwin Biotech (Plantation, FL). N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) is from Pierce Chemicals (Rockford, IL). Sodium phosphate, sodium chloride and EDTA are from Fisher Scientific (Pittsburgh, PA). Sephadex G-25 gel permeation matrix (fine grade), Superose S-200 FPLC and Blue Sepharose 6 Fast Flow are from GE Healthcare (Piscataway, NJ). Dithiothreitol (DTT), iodoacetamide and dimethylformamide (DMF) are from Sigma (St. Louis, MO). Bradford protein assay reagent was from Bio-Rad (Hercules, CA). Acrodisc with Mustang Q membrane units are from Pall Corp. (Ann Arbor, MI). QCL-1000 endotoxin detection kits are from Lonza (Walkersville, MD). Alexa Fluor 488 goat anti-rabbit IgG was purchased from Invitrogen (Grand Island, NY). FITC-coupled anti-rabbit IgG was from Sigma (St. Louis, MO). Horseradish peroxidase–conjugated goat anti-rabbit IgG was purchased from Bio-Rad (Hercules, CA).

Cell lines and cell culture

Cell lines were obtained from the American Type Culture Collection and maintained in either DMEM (Capan-1, Capan-2, L3.6P1, AsPc1, MIA PaCa-2, U-87 MG cells), DMEM/F12 (MDA-MB-231, Eb-1, Calu-3 and RAW264.7 cells), RPMI 1640 (MDA-MB-435, MCF7, BT474, BxPc-3, NCI-N87 and Jurkat cells), McCoy’s 5A (T-24, HT-29, SKOV3, ME-180 and SKBR3 cells), F12 (PC-3) or Eagle’s MEM (HT-1080) medium. Fn14-deficient mouse embryonic fibroblasts (MEF 3.5-/-) were maintained in DMEM. All
media contained 10% fetal bovine serum. Cells were grown at 37°C with 5% CO₂ at constant humidity. Media and supplements were purchased from Invitrogen (Grand Island, NY).

**Construction and purification of the ITEM4-rGel conjugate**

A 3-fold molar excess of the cross-linker N-succinimidyl-3-(2-pyridylodithio) propionate (SPDP; Pierce, Rockford, IL) was added to 2 mL of ITEM-4 (5 mg/ml in PBS), and allowed to react for 30 min at room temperature. Excess, unreacted SPDP was removed by gel filtration using Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden) gel chromatography. Recombinant gelonin (5-fold molar excess versus ITEM-4) was reduced by adding 2 mM DTT (Sigma, St. Louis, MO) and stirring for 30 min at room temperature. Excess, unreacted DTT was removed by Sephadex G-25 gel chromatography. ITEM4-SPDP was slowly added to the rGel-DTT, with stirring, and the conjugation was allowed to proceed for 6 h at 4 °C under N₂ gas. Iodoacetamide (Sigma) was then added to a concentration of 2 mM to block any remaining, unconjugated ITEM-4. Unconjugated rGel was removed by passage through a Superose S-75 fast protein liquid chromatography column (Amersham). The concentration of NaCl was reduced to <10 mM by dilution, and the conjugate was applied to a Blue Sepharose (Amersham) column. Unconjugated ITEM-4 was eluted by washing with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.2), and the conjugate was eluted with 10 mM sodium phosphate, 2 M NaCl. The purified conjugate was dialyzed into PBS and concentrated using an Amicon Ultra filter (Millipore, Billerica, MA). Endotoxin levels were determined with the QCL-1000 kit (Lonza Inc, Walkersville, MD), according to the
manufacturer’s instructions. If the level was above 50 EU/mg protein, the sample was slowly passed through an Acrodisc Mustang Q membrane, and the endotoxin levels were then re-assessed. The final conjugate preparations were then aliquoted and stored at -20 °C.

**Flow cytometry**

To analyze Fn14 cell surface expression and cell binding activity of ITEM4-rGel, flow cytometric analysis of cells stained with ITEM-4 or ITEM4-rGel was performed as previously described (18). Briefly, 5×10^5 cells were incubated for 1 h on ice with ITEM-4, ITEM4-rGel (2 μg/100 μl in 1% BSA in PBS), or mouse IgG2a isotype control all at the same molar concentrations. Cells were then washed twice with 0.5% BSA in PBS and incubated for an additional 30 min on ice with a FITC-conjugated goat anti-mouse IgG mAb. Following two washes, cells were fixed in 3.7 % paraformaldehyde and analyzed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, Mountain View, CA).

**Surface plasmon resonance assay**

Binding of ITEM-4 and ITEM4-rGel to immobilized, recombinant Fn14 extracellular domain (Cell Sciences) was measured using a BIACore 3000 instrument as previously described (19). The binding to a blank cell (non-specific binding) was subtracted from the sensogram.

**ITEM4-rGel internalization assay**
Human T-24 bladder tumor cells were plated onto polylysine-coated 16-well chamber slides (Nunc, Rochester, NY) at a density of $1 \times 10^4$ cells per well and treated with 50 nM rGel for 8 h or ITEM4-rGel for 2 or 8 h. Proteins bound to the cell surface were removed by incubation with glycine buffer (500 mM NaCl and 0.1 M glycine, pH 2.5) and neutralization for 5 min with 0.5 mol/L Tris (pH 7.4) followed by a wash with PBS. Blocking of non-specific protein binding sites and immunofluorescence staining followed by visualization using a laser scanning confocal microscope were performed as previously described (18).

**Cytotoxicity assays**

Cytotoxicity of ITEM4-rGel and rGel against various tumor cells was assessed as previously described (18). Log-phase cells were plated in 96-well flat-bottomed tissue culture plates and allowed to adhere overnight. Purified ITEM4-rGel, rGel and/or ITEM-4 were diluted in culture medium and added to the wells in 5-fold serial dilutions. Cells were incubated for 72 h. The remaining adherent cells were stained with crystal violet (0.5% in 20% methanol) and solubilized with Sorenson’s buffer (0.1 M sodium citrate, pH 4.2, in 50% ethanol). Absorbance was measured at 595 nm. Competition cytotoxic assays were performed by plating SKOV3 cells in 96-well plates and by preincubating the cells for 2 h with 1 μM ITEM-4 before addition of 5-fold serial dilutions of ITEM4-rGel. Results were analyzed by staining the remaining adherent cells with crystal violet as described above.

**Apoptosis assays**
Apoptosis was assessed using the Annexin V-FITC Kit (Molecular Probes, Inc. Eugene, OR) to distinguish cells that were in the early apoptosis (annexin V+/PI-) or late apoptosis/necrosis (annexin V+/PI+) phase (20). Apoptosis induction through mitochondrial membrane depolarization was also investigated using the cationic dye JC-1 (MitoProbe, JC-1 Assay Kit) according to manufacturer’s instructions.

**Lactate dehydrogenase (LDH) release assay**
LDH was measured using LDH Cytotoxicity Detection Kit from Clontech Laboratories, Inc. (Mountain View, CA) according to manufacturer’s instructions.

**Analysis of high-mobility group B1 (HMGB1) cellular release**
Whole-cell lysates were resolved on 4–12% Criterion XT Bis-Tris gels (Bio-Rad, Hercules, CA) and transferred to a nitrocellulose membrane as previously described (18). After blocking, the membrane was incubated overnight at 4 °C with anti-HMGB1 antibody (Santa Cruz Biotechnologies, sc-74085). After incubation with peroxidase-conjugated secondary antibodies for 1 h at room temperature, signals were visualized by enhanced chemiluminescence (Pierce, Rockford, IL) according to the manufacturer’s instructions. Levels of HMGB1 in the culture medium were determined by Western blot analysis as previously described (21).

**Xenograft tumor model and localization of ITEM4-rGel after systemic administration**
Animal procedures were performed according to a protocol approved by the AALAC-approved Animal Care and Use Facility at M.D. Anderson Cancer Center. Athymic, female nu/nu mice (8 weeks old) were obtained from Taconic Farms. Human T-24 bladder tumor cells ($5 \times 10^6$) were suspended in 100 $\mu$L of PBS mixed with 100 $\mu$L Matrigel (Becton Dickinson, Franklin Lakes, NJ) and then subcutaneously injected (hind flank) into the mice. Tumor volumes were determined by the formula: volume = length $\times$ width $\times$ height. When tumors reached a mean volume of ~50-100 mm$^3$, mice were randomized (five per group) before injecting ITEM4-rGel or PBS (i.v., tail vein) every six days for 30 days with a total dose of 20 and 30 mg/kg. Tumor growth was measured with calipers every ~7 days. Data are presented as mean ± SEM. P values were obtained using a Student’s 2-tailed t test with 95% confidence interval for evaluation of the statistical significance compared with the controls. A value of $p < 0.05$ was considered statistically significant.

Another group of mice bearing T-24 xenograft tumors were administered ITEM4-rGel (200 $\mu$g/mouse) and PBS. Twenty-four hours later, animals were euthanized and tumor tissue was removed, snap-frozen and sectioned. To examine the presence of ITEM4-rGel, the sections were dried and then fixed in 3.7% formaldehyde (Sigma, St. Louis, MO) for 20 min at RT followed by a brief rinse with PBS. Cells were then permeabilized for 10 min in PBS containing 0.2% Triton X-100, washed three times with PBS, and blocked with PBS containing 3% bovine serum albumin for 1 h at RT. Fixed cells were incubated with rabbit anti-rGel antibody (22) for 2 h at RT. The slides were washed with PBS and then incubated with anti–rabbit IgG–FITC–conjugated antibody. Cell nuclei were counterstained by exposure to propidium iodide (PI, 1 $\mu$g/ml) for 1 h at...
RT. After a final wash step, the slides were mounted and analyzed under a fluorescence microscope.

**Terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay to detect apoptosis**

The T-24 tumor frozen sections were stained by TUNEL using an in situ cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer’s instructions. Samples were analyzed under a Nikon Eclipse TS100 fluorescent microscope (Tokyo, Japan), and photographs were taken with a scope-mounted Nikon digital camera.
Results

Preparation of ITEM4-rGel immunoconjugate

We utilized the high affinity, murine anti-Fn14 monoclonal antibody ITEM-4 (3) to generate a chemical conjugate with recombinant rGel toxin (designated ITEM4-rGel) using the heterobifunctional cross-linker SPDP as described in Materials and Methods. The ITEM4-rGel conjugate was purified and the final product was found to contain no contaminating free antibody or rGel as shown in Fig. 1A. Analysis of the preparation confirmed that the final material contained both antibody + 1 rGel (major) and antibody + 2 rGel (minor) species (Fig. 1B).

The TWEAK receptor Fn14 is overexpressed in multiple tumor cell lines

We next examined Fn14 expression in a panel of normal and tumor cell lines by both Western blot analysis and flow cytometry. Fn14 expression was detected by Western blotting in 17/21 tumor cell lines tested (Fig. 2A). A mouse macrophage cell line (RAW264.7) and an embryonic fibroblast cell line generated from Fn14-deficient mice did not express Fn14. A similar pattern of Fn14 expression was found by flow cytometry using the ITEM-4 monoclonal antibody (Fig. 2B and Table 1). These results demonstrate that a large number of tumor cell lines representing multiple tumor types constitutively express the TWEAK receptor Fn14.
ITEM4-rGel specifically binds to the Fn14 receptor and is internalized into Fn14-positive T-24 cells

To examine whether modification of the ITEM-4 antibody through the conjugation process affected the binding to Fn14, ITEM-4 and ITEM4-rGel were tested for binding to both recombinant Fn14 and Fn14-expressing cells. ITEM-4 and ITEM4-rGel binding to recombinant Fn14 extracellular domain was determined by surface plasmon resonance analysis using a BIAcore instrument. We found that ITEM-4 and ITEM4-rGel bound to Fn14 with similar equilibrium dissociation constants (Kₐ) of ~1.1 nM and 0.7 nM, respectively (Fig. 3A). Purified rGel did not bind to Fn14 in this assay. We could find no differences in the flow cytometry binding curves for ITEM-4 (Fig. 2B) or ITEM4-rGel (Fig. 3B) using Fn14-expressing cells (MDA-MB-231, T-24, HT-29) as targets. Although it is known that ITEM-4 recognizes the murine Fn14 receptor (23) neither antibody nor immunoconjugate were found to bind to Fn14-deficient MEF cells as determined by flow cytometry (Fig. 2B and 3B). These data show that the binding affinity and the selectivity of the native ITEM-4 antibody appeared to be unaffected by conjugation to the rGel toxin.

We next examined the ability of ITEM4-rGel to internalize and deliver rGel to the cytoplasm of Fn14-expressing T-24 tumor cells. Immunofluorescence studies showed that exposure of cells to ITEM4-rGel resulted in efficient, rapid internalization of the rGel component to the cytoplasm (Fig. 3C). It is important to note that in processing treated cells for internalization analysis, the use of brief acid exposure to remove surface-bound immunoconjugate permits preferential detection of internalized conjugate. When these cells were treated with rGel alone, no internalization was detected (Fig. 3C). In addition,
ITEM4-rGel internalization was not observed when the conjugate was added to Fn14-deficient mouse embryonic fibroblasts (data not shown).

**ITEM4-rGel is highly cytotoxic to Fn14-expressing tumor cells**

We examined the cytotoxic effects of ITEM4-rGel on cell lines expressing various levels of Fn14 to determine the general sensitivity of Fn14-expressing tumor cells and to correlate the cytotoxic effects of the immunoconjugate to Fn14 expression levels. The targeting index (the ratio of IC$_{50}$ for rGel vs the IC$_{50}$ for ITEM4-rGel) was calculated for each cell line. This ratio represents the ability of the ITEM-4 component of the ITEM4-rGel immunoconjugate to mediate delivery of the rGel toxin component to the target cell cytoplasm and normalizes for the inherent cellular sensitivity to the rGel toxin. As shown in Table 1, the highest targeting index was found in breast, pancreatic and melanoma tumor lines. The breast tumor cell line eB1 was found to be the most sensitive to ITEM4-rGel (targeting index = 83,500). A number of cell lines which express low levels of Fn14 (eB1, U87-MG and BT474) were nevertheless sensitive to ITEM4-rGel treatment. Overall, ITEM4-rGel was shown to be 8 - 8 x10$^4$ fold more potent than free rGel. Cell lines which were negative for Fn14 expression were not sensitive to the conjugate.

The cytotoxicity profile of ITEM4-rGel, rGel and ITEM-4 following addition to MDA-MB-231, HT-29, T-24, BxPC-3, MDA-MB-435 and Fn14-deficient MEF cells in culture is shown in Figure 4A. ITEM4-rGel was able to specifically kill Fn14-expressing cells in a dose-dependent manner, whereas native ITEM-4 antibody alone had no effect at doses of up to 1 μM. By comparison, the cytotoxic effects of ITEM4-rGel on non-target MEF cells were similar to the free rGel with a targeting index of 1.
To confirm that Fn14 binding was required for cytotoxicity of the conjugate, we first pre-incubated SKOV3 cells with 1 μM ITEM-4 for 2 h before addition of various amounts of ITEM4-rGel. As shown in Figure 4B, ITEM4-rGel was cytotoxic to SKOV3 cells with an IC50 value of 0.4 nM. Pre-incubation with 1 μM ITEM-4 for 2 h completely abrogated ITEM4-rGel-induced cytotoxicity thereby confirming that initial binding of ITEM4-rGel to the Fn14 receptor is required to initiate the cytotoxic effects of the conjugate.

We next examined the minimal contact time required for the ITEM4-rGel conjugate to generate the optimum biological effect. We exposed MDA-MB-231 and T-24 cells to various ITEM4-rGel concentrations for various times ranging from 1 to 72 h. As summarized in Table 2, the lowest IC50 doses were observed after 24 h of exposure and there was no appreciable increase in sensitivity of cells for longer exposure times.

**ITEM4-rGel treatment induces apoptosis characterized by HMGB1 release**

The mechanisms responsible for the cytotoxicity of ITEM4-rGel were investigated using human T-24 bladder carcinoma cells. These cells were treated with 1 nM rGel, ITEM-4 or ITEM4-rGel for 72 h and then subjected to Annexin V and PI staining. Treatment with ITEM4-rGel resulted in a significant increase in Annexin V-positive cells suggesting that the conjugate promotes target cell apoptosis (Fig. 5A). We also demonstrated that exposure of T-24 cells to ITEM4-rGel, but not ITEM-4 or rGel, resulted in mitochondrial membrane depolarization using the mitochondrial dye JC-1 (Fig. 5B). This finding is also consistent with an apoptotic cell death mechanism.
It has been reported that most forms of tumor cell death result in release of the RAGE ligand high-mobility group protein 1 (HMGB1) (21). Therefore, we tested whether HMGB1 would be released from tumor cells following treatment with ITEM4-rGel. We treated T-24 cells with either rGel, ITEM-4 or ITEM4-rGel for 24 h and assessed HMGB1 levels in cell lysates and conditioned media by Western analysis. We observed that treatment of cells with both ITEM-4 and ITEM4-rGel resulted in HMGB1 release (Fig. 5C). HMGB1 release usually occurs in cells that are undergoing classical necrotic cell death marked by abrupt membrane lysis and the release of soluble proteins (24). To evaluate the release of another soluble protein, we measured lactate dehydrogenase (LDH) release in treated T-24 cells and found that neither ITEM-4 nor ITEM4-rGel treatment resulted in LDH release (Fig. 5D). Treatment of cells with the ITEM-4 antibody did not have demonstrable cytotoxic effects nor did it cause measurable apoptotic damage. These data indicate that HMGB1 release from ITEM4-treated cells did not appear to be associated with necrosis but instead may represent selective release of the HMGB1 protein from cells.

ITEM4-rGel localizes in T-24 cell xenograft tumors after intravenous administration

We next investigated whether the ITEM4-rGel conjugate was delivered to xenograft tumors following i.v. injection. The ITEM4-rGel conjugate or PBS were injected into mice bearing T-24 bladder tumors and the tumors were harvested 24 h later. Tumor sections were made and assayed for the presence of ITEM4-rGel by immunofluorescence using an anti-rGel antibody. As shown in Figure 6A, tumors obtained from the ITEM4-
rGel-injected mice showed staining with the rGel antibody while there was no staining observed in the saline-treated group. In general, the intratumoral localization of the ITEM4-rGel conjugate (green) appeared to be relatively uniform throughout the tumor sections stained although there were a few areas of intense staining noted.

**Inhibition of tumor growth in vivo by ITEM4-rGel**

We then determined whether ITEM4-rGel administration could inhibit the growth of T-24 bladder tumor xenografts growing subcutaneously (flank) in nude mice. T-24 cells were injected into mice and when tumors reached a mean volume of ~50-100 mm$^3$ mice were randomized (five per group) before injecting either ITEM4-rGel or PBS (i.v., tail vein) every six days for 30 days with a total dose of 20 or 30 mg/kg. As shown in Fig. 6B, tumor volumes in the saline-treated control mice increased ~16-fold (from ~50 mm$^3$ to a mean of 800 mm$^3$) over the 125 day course of the experiment. In contrast, tumors from the ITEM4-rGel treated mice increased ~3.6-fold (from ~50 mm$^3$ to a mean of 182 mm$^3$) and showed no change in tumor size over the same period for 20 mg/kg and 30 mg/kg dosage groups, respectively. There were no animal deaths in the studies and mouse body weights showed no significant change in any of the treated or control groups over the duration of the experiment (data not shown). We next examined tumors from the control and treated groups. Histological (H&E) stain of tumors from the control and treated groups at 125 days demonstrated viable tumor cells in all groups (data not shown). However, as shown in Figure 6C, there was a significant increase in the number of apoptotic cells (as assessed by TUNEL staining) in tumors from mice treated with ITEM4-rGel.
Discussion

Various TNF and TNFR superfamily members have been identified as being essential elements in tumor growth and development (25-27). In addition, they have also been implicated in the response and resistance of tumor cells to therapeutic agents (28, 29). For many of these reasons, TNF family member ligands and their cognate receptors appear to be attractive targets for intervention and for the development of targeted therapeutics for the treatment of cancer and immunologic diseases (28, 30, 31). One particular TNF:TNFR pair, TWEAK and Fn14, has been implicated in several important processes associated with tumor growth and metastasis (5-13, 32, 33) and a recent report has shown that administration of an anti-Fn14 mAb can inhibit tumor growth in xenograft assays (8).

The use of antibodies to interfere with growth factors and their receptors present on tumors has gained wide acceptance as a therapeutic strategy (34-36). Targeting the HER1, HER2 and VEGF pathways with antibodies is now considered an essential component in the clinical therapeutic management of a variety of tumor types (37). There are also numerous groups developing antibody conjugates for the directed delivery of highly cytotoxic payloads such as small molecules (auristatin, calicheamycin, or maytansinoids (38-41), protein toxins (RTA, PE, gelonin (22, 42, 43)) or other cytotoxic human enzymes such as serine proteases (44). The current study demonstrates a unique Fn14 receptor-targeted antibody-toxin conjugate designated ITEM4-rGel which is shown to be capable of specifically delivering rGel to Fn14-expressing tumor cells in vitro and in vivo. As described previously, activation of TWEAK signaling by either ligands or antibodies have the potential to elicit pro-growth effects in addition to defined cytotoxic properties. The use of cytotoxic conjugates targeting Fn14 have the potential to augment
the cytotoxic effects of pathway activation while eliminating the growth stimulatory properties.

The antibody ITEM-4 and its corresponding immunoconjugate ITEM4-rGel was shown to selectively bind the Fn14 receptor on tumor cells. The cytotoxicity of ITEM4-rGel on Fn14-expressing tumor cells was investigated extensively in vitro. ITEM4-rGel was found to be selectively toxic to a wide range of human tumor cell lines expressing the Fn14 receptor (Table 1). Cells over-expressing Fn14 were highly sensitive to the ITEM4-rGel, whereas cells expressing no Fn14 were no more sensitive to the conjugate than they were to free rGel. Surprisingly, some cell lines expressing high (Calu-3) or intermediate (ME-180, HT-29) levels of Fn14 showed relatively low levels of sensitivity to the construct. Alternatively, eB1 breast tumor cells expressing low levels of Fn14 showed extreme sensitivity to the construct. The observed differences in cellular response to the conjugate may be the result of differences in cellular protein synthesis rates, receptor recycling, immunotoxin internalization efficiency (45, 46) or intracellular trafficking and release from endosomal compartments (47). All of these factors may play a role in determining the ultimate response of cells to the ITEM4-rGel construct.

Our previous studies with rGel-based constructs showed that some constructs induced apoptosis (48), but some did not and this appeared to be highly dependent on the cell type under investigation (49). We found that the ITEM4-rGel induced cell death with clear mitochondrial-dependent apoptosis. We additionally showed that when T-24 cells were treated with either ITEM-4 alone or ITEM4-rGel, the protein HMGB1 was released into the media. The effect of unconjugated ITEM-4 on HMGB1 release was unexpected since ITEM-4 showed no effect on the growth of these cells in culture. The effect of
ITEM-4 on HMGB1 release by tumor cells has not been reported previously, but a study by Kalinina et al. (50) reported that treatment with the Fn14 ligand TWEAK caused an upregulation of HMGB1 synthesis and secretion by normal cells as part of the inflammatory process. It is possible that ITEM-4 agonistic effect on the Fn14 receptor could cause release of HMGB1 in certain cell types.

Fn14 is gathering much attention at this time due to its strong overexpression in many of the most prevalent and deadly solid tumor types. We utilized the T-24 bladder model because the in vitro response to the cytotoxic effects of the conjugate and the relative expression levels of the Fn14 receptor (via Western blot) appeared to be intermediate in the range of cell lines tested. The results of our studies using the T-24 xenograft model show that an immunoconjugate targeting Fn14 can suppress tumor growth. The maximum tolerated dose (MTD) for ITEM4-rGel with this schedule was determined to be 65 mg/kg (data not shown) and we selected 20 and 30mg/kg total dosages (30 and 50% of the MTD respectively) for these initial studies. The in vivo data shows that effective tumor cell killing (by induction of apoptosis as assayed by TUNEL assay) can be achieved with ITEM4-rGel at these doses without any observable side effects in mice suggest that targeting of the conjugate to normal mouse tissues is minimal. The IHC studies demonstrated that ITEM4-rGel uniformly distributed in tumor xenografts after i.v. administration although additional studies are ongoing to examine the pharmacokinetics and the relative uptake kinetics of ITEM4-rGel into tumor compared to normal organs. The results from these studies may define a new schedule of administration to optimize tumor uptake of the immunoconjugate.
In summary, this study demonstrates a proof of concept that Fn14 appears to have an excellent potential for targeted therapy approaches and ITEM-4-based therapeutic agents appear to warrant further development. While therapeutic studies with ITEM4-rGel demonstrate \textit{in vivo} efficacy, generating a human or humanized version of ITEM-4 is essential for long-term clinical administration. The immunogenicity of the rGel component of the conjugate is of potential concern although clinical studies of an immunoconjugate with the anti-CD33 antibody HuM195 have demonstrated limited antigenicity of the rGel component even with repeated administration (Cortes \textit{et al.}, unpublished). Studies of anti-Fn14 constructs containing second-generation payloads such as de-immunized rGel toxin (designated drGel) or fully human human granzyme B as a replacement for full-length rGel are in progress.
Acknowledgments

We thank Sharron Brown and Molly Migliorini for conducting the surface plasmon resonance (Biacore) analysis and Rebeca Galisteo for performing preliminary immunoconjugate in vivo efficacy experiments.
Reference List


7. Tran NL, McDonough WS, Savitch BA, Sawyer TF, Winkles JA, Berens ME. The tumor necrosis factor-like weak inducer of apoptosis (TWEAK)-fibroblast growth factor-inducible 14 (Fn14) signaling system regulates glioma cell survival


Table 1. Fn14 status and cytotoxic effect of ITEM4-rGel on various normal and tumor cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Tumor types</th>
<th>Fn14 receptor</th>
<th>rGel IC₅₀ (nM)</th>
<th>ITEM4-rGel IC₅₀ (nM)</th>
<th>Targeting Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB1</td>
<td>breast</td>
<td>+</td>
<td>835</td>
<td>0.01</td>
<td>83,500</td>
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<td>MDA-MB-231</td>
<td>breast</td>
<td>+++</td>
<td>717.7</td>
<td>0.08</td>
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<td>MCF-7</td>
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<td>+++</td>
<td>237.4</td>
<td>0.03</td>
<td>7,913</td>
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<td>SKBR3</td>
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<td>++</td>
<td>2,712</td>
<td>10.6</td>
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<td>BT-474</td>
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<td>--</td>
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<td>CaPan-2</td>
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<td>++++</td>
<td>2270</td>
<td>0.1</td>
<td>22,700</td>
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<tr>
<td>MIA-PaCa-2</td>
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<td>638.7</td>
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<td>AsPC-1</td>
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<td>3980</td>
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<td>++</td>
<td>295.6</td>
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<td>L3.6P1</td>
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<td>++</td>
<td>230</td>
<td>0.6</td>
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<td>974.9</td>
<td>7.3</td>
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<td>ME-180</td>
<td>cervix</td>
<td>+++</td>
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<tr>
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<td>Mouse</td>
<td>-</td>
<td>172.7</td>
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<tr>
<td>MEF 3.5/-</td>
<td>Mouse</td>
<td>-</td>
<td>652.7</td>
<td>514.5</td>
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</tbody>
</table>

*Targeting Index is defined as: (IC₅₀ of rGel)/(IC₅₀ of ITEM4-rGel).

NOTE: Fn14 expression is based on the percentage of cells stained with ITEM-4 detected by flow cytometry. -, negative; +, <10% positive; 11% to 25% positive; ++, 26% to 50% positive; ++++, 51% to 75% positive; ++++, >75% positive.
Table 2. In vitro cytotoxicity of ITEM4-rGel and exposure duration

<table>
<thead>
<tr>
<th>Drug exposure time (h)</th>
<th>IC$_{50}$ (nM)</th>
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<tr>
<td></td>
<td>T-24</td>
</tr>
<tr>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>2.4</td>
</tr>
<tr>
<td>8</td>
<td>0.2</td>
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<tr>
<td>24</td>
<td>0.03</td>
</tr>
<tr>
<td>48</td>
<td>0.04</td>
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<tr>
<td>72</td>
<td>0.07</td>
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</table>
Figure Legends

Figure 1. ITEM4-rGel conjugate preparation and purification. A. SDS-PAGE analysis of the purified ITEM-4, rGel and ITEM4-rGel immunoconjugate on 10% non-reduced gel. B. SDS-PAGE analysis of ITEM4-rGel with different loading volumes on 6% non-reduced gel. The resultant ITEM4-rGel was composed of antibody + 1 rGel (predominant) and antibody + 2 rGel (minor) species and was essentially free of contaminating rGel or unreacted ITEM-4 antibody.

Figure 2. Fn14 receptor expression in various tumor cell lines. A. Expression of Fn14 protein by 21 different human tumor cell lines was assayed by Western blot using anti-Fn14 monoclonal antibody ITEM-4. β-actin was used as a control for protein loading. B. Fn14 expression analysis by flow cytometry. MDA-MB-231, T-24, HT-29 and MEF 3.5-/- cells were incubated with ITEM-4 followed by FITC-conjugated goat anti-mouse IgG antibody and analyzed with a FACSCalibur flow cytometer. Black curve, isotype control (cells+mouse IgG2a +FITC-Ab); red curve, Fn14 (cells+ ITEM-4 + FITC-Ab).

Figure 3. ITEM4-rGel specifically binds and internalizes into Fn14-expressing tumor cells. A. Surface plasmon resonance analysis of ITEM-4 and ITEM4-rGel binding to immobilized recombinant Fn14 extracellular domain. B. MDA-MB-231, T-24, HT-29 and MEF 3.5-/- cells were incubated with ITEM4-rGel followed by FITC conjugated goat anti-mouse IgG antibody and analyzed with a FACSCalibur flow cytometer. Green curve, Untreated cells; Blue curve, isotype control (cells + mouse IgG + FITC-Ab); red curve, ITEM4-rGel (cells + ITEM4-rGel + FITC-Ab). C. T-24 cells were either left untreated or treated with either 50 nM ITEM4-rGel for 2 and 8 h or rGel for 8 h. The cells were fixed, acid washed to remove surface-bound material,
permeabilized, and immunostained for the presence of rGel using a rabbit anti-rGel antibody (green). The cells were counterstained with propidium iodide (red) to identify nuclei and visualized using a laser scanning confocal microscope.

**Figure 4.** Cytotoxicity of ITEM4-rGel when added to different tumor cell lines. **A.** Different concentrations of ITEM-4, rGel and ITEM4-rGel were added to various tumor cell lines (MDA-MB-231, HT-29, T-24, BxPC-3, MDA-MB-435) and Fn14-deficient mouse embryonic fibroblasts (MEF 3.5/-) and cytotoxicity was measured as described in Materials and Methods. **B.** SKOV3 cells were either left untreated or incubated with different concentrations of ITEM-4, ITEM-4-rGel or rGel for 72 h or pre-treated with 1 μM ITEM-4 for 2 h, and then co-incubated with different concentrations of ITEM4-rGel for another 72 h. Cytotoxic effects were assessed as above.

**Figure 5.** Analysis of ITEM4-rGel-induced cell death. **A.** T-24 cells were either left untreated or treated with rGel, ITEM-4, or ITEM4-rGel for 72 h and then stained with Alexa Fluor® 488 annexin V and propidium iodide, followed by flow cytometry analysis. *Numbers in the quadrants,* percentage of cells in each category. **B.** T-24 cells were either left untreated or treated with rGel, ITEM-4 or ITEM4-rGel for 72 h and then mitochondrial membrane depolarization was assayed by JC-1 staining followed by flow cytometry analysis. *Numbers in the quadrants,* percentage of cells of each category. **C.** Western blot analysis of cell extract and conditioned media for HMGB1 protein after treatment of T-24 cells with rGel, ITEM-4 or ITEM4-rGel for 24 h. **D.** T-24 cells were treated with different concentrations of rGel, ITEM-4 and ITEM4-rGel for 24 h and LDH release was measured. Data shown is mean ± S.D. from three replicates.
Values for medium alone represent baseline LDH for culture media containing 1% FBS. Values for cells in media represent untreated cells. Values for Triton X-100 represent the maximal release of LDH from the cells after detergent treatment.

**Figure 6.** ITEM-4-rGel administered to mice localizes in tumor tissue and inhibits tumor growth.  

**A.** ITEM4-rGel was administered (*i.v.*) to mice bearing T-24 bladder carcinoma xenograft tumors. One day later, animals were sacrificed, and tumor tissues were removed, fixed, sectioned, and assayed for the presence of ITEM4-rGel by immunofluorescence using a rabbit anti-rGel antibody (green). The cells were counterstained with propidium iodide (red) to identify nuclei and visualized using a Nikon Eclipse TS 100 fluorescent microscope.  

**B.** T-24 cells were injected subcutaneously into mice and tumors were allowed to grow to 50-100 mm$^3$ in volume. Mice (n=5) were injected (*i.v.*, tail vein) with either saline (control) or the ITEM4-rGel conjugate six times at 20 mg/kg or 30 mg/kg total dose. Data represent mean tumor volumes ± SEM, and asterisks indicate significant difference (*P* <0.05) when comparing ITEM4-rGel treated mice vs. the control group.  

**C.** Apoptosis detection in tumor tissue by TUNEL assay. Mice bearing T-24 xenograft tumors were administered intravenously with either saline (control) or the ITEM4-rGel (20 and 30 mg/kg). Tumor tissue sections were stained by TUNEL and analyzed under a Nikon Eclipse TS 100 fluorescent microscope.
Figure 1

A

B

ITEM4  
1 2 5

ITEM4-rGel  
10 5 2 1

(μL) Marker

x 2 rGel
x 1 rGel

- 150 KDa
- 100 KDa

150 KDa -
100 KDa -
75 KDa -
50 KDa -
35 KDa -
25 KDa -
15 KDa -
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>HT-29</th>
<th>N-87</th>
<th>SKOV3</th>
<th>T-24</th>
<th>U-87 MG</th>
<th>PC-3</th>
<th>Jurkat</th>
<th>RAW264.7</th>
<th>MEF3.5-/-</th>
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<th>SKBR3</th>
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</tr>
</tbody>
</table>

B

- Isotype
- MDA-MB-231
- T-24
- HT-29
- MEF3.5-/-
Figure 3

A

B

C

Non-treated  rGel, 8 h  ITEM4-rGel, 2 h  ITEM4-rGel, 8 h
Figure 4

A

Cytotoxicity of ITEM4-rGel on MDA-MB-231

Cytotoxicity of ITEM4-rGel on HT-29

Cytotoxicity of ITEM4-rGel on T-24

Cytotoxicity of ITEM4-rGel on BxPC-3

Cytotoxicity of ITEM4-rGel on MDA-MB-435

Cytotoxicity of ITEM4-rGel on MEF 3.5 -/-

B

Competitive cytotoxic assay on SKOV3
Figure 6

A

Saline

ITEM4-rGel

Anti-rGel

PI (Nucleus)

Merge (200×)

B

Saline

ITEM4-rGel (30 mg/kg)

ITEM4-rGel (20 mg/kg)

C

Saline (control)

ITEM4-rGel (20 mg/kg)

ITEM4-rGel (30 mg/kg)
Molecular Cancer Therapeutics

Development and Characterization of a Potent Immunoconjugate Targeting the Fn14 Receptor on Solid Tumor Cells

Hong Zhou, John W. Marks, Walter N Hittelman, et al.

Mol Cancer Ther Published OnlineFirst May 17, 2011.

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doi:10.1158/1535-7163.MCT-11-0161

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