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Genetically designing a more potent anti-pancreatic cancer agent by simultaneously cotargeting human IL-13 and EGF receptors in a mouse xenograft model

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Abstract

Objective—Investigators are currently interested in the EGFR and IL-13R as potential targets in the development of new biologicals for pancreatic cancer. Attempts to develop successful agents have met with difficulty. Our novel approach was to simultaneously target these receptors with EGF and IL-13 cloned on the same bispecific single chain molecule with truncated diphtheria toxin (DT₃₉₀) to determine if co-targeting with DTEGF13 had any advantages.

Design—Proliferation experiments were performed to measure the potency and selectivity of bispecific DTEGF13 and its monospecific counterparts against pancreatic cancer cell lines Panc-1 and MiaPaCa-2 in vitro. DTEGF13 was then administered intratumorally to nude mice with MiaPaCa-2 flank tumors to measure efficacy and toxicity (weight loss).

Results—In vitro, bispecific DTEGF13 was 2,800-fold more toxic than monospecific DTEGF or DTIL13 against Panc-1. A similar enhancement was observed in vitro when MiaPaCa-2 pancreatic cancer cells or H2981-T3 lung adenocarcinoma cells were studied. DTEGF13 activity was blockable with recombinant EGF13. DTEGF13 was potent (IC₅₀ = 0.00017 nM) against MiaPaCa-2, receptor specific, and significantly inhibited MiaPaCa-2 tumor in nude mice (p<0.008).

Conclusions—In vitro studies show that the presence of both ligands on the same bispecific molecule is responsible for the superior activity of DTEGF13. Intratumoral administration showed that DTEGF13 was highly effective in checking aggressive tumor progression in mice. Lack of weight loss in these mice indicated that the drug was tolerated and a therapeutic index exists in an “on target” model in which DTEGF13 is cross-reactive with native mouse receptors.

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Competing Interests

The authors have no competing interests to disclose in the submission and publication of this work.

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Keywords

carcinoma; cytotoxin; diphtheria toxin; EGF; IL-13

Introduction

Pancreatic adenocarcinoma is one of the most lethal cancers. Only 10–15% of patient's tumors are resectable at diagnosis because of aggressive growth (1). More than 32,000 patients each year die of this cancer in the US alone mostly due to its highly aggressive and rapid metastasis (2). Pancreatic cancer is most commonly treated with the nucleoside analog Gemcitabine, but the median survival time is less than 6 months (3,4)_[sbl1]. Studies have shown that pancreatic tumors overexpress EGFR (5) and others have shown that fusion toxins targeting the EGFR show promise for pancreatic cancer therapy (6–9). Also, IL-13R is expressed on pancreatic tumors. For example, investigators showed that 6 of 6 pancreatic cancer cell lines examined expressed IL-13R alpha 1 and IL-4R alpha, one cell line expressed IL-13R alpha 2, and 5 pancreatic cancer cell lines expressed gamma c (10). IL-13R-directed fusion toxins also have successfully been shown to kill pancreatic tumor cells (10,11).

Epidermal growth factor (EGF) is the main ligand of the epidermal growth factor receptor (EGFR), a transmembrane signaling protein from the erbB family (12). Studies have revealed a link between EGFR signaling pathways and malignancy (13). Interleukin-13 (IL-13) (14, 15) secreted by activated Type-2 T cells and mast cells (16), is a pleiotropic lymphokine regulating inflammatory and immune responses. It modulates human monocyte and B cell functions but not those of T cells (17). Interleukin-13 receptors (IL-13R) are found to be overexpressed on solid tumor cells including glioblastoma (18–21), renal cell carcinoma (22), AIDS Kaposi's sarcoma (23), and cancers of prostate (24), ovary (25), and head and neck (26). IL-13 has proven a useful ligand for therapy because along with IL-13R being overexpressed on tumors, its expression on normal cells is limited to B cells and monocytes. It appears that IL-13R may function as a tumor-specific, high affinity target and incorporating IL-13 into a cytotoxin (CT) may be a beneficial strategy.

In this study, we created a novel single-chain recombinant bispecific cytotoxin (CT) by linking a fragment of diphtheria toxin (DT) to human EGF and IL-13 forming the fusion protein DTEGF13. Diphtheria toxin is used for cytotoxin construction because a single molecule delivered to the cytosol is sufficient to bring about cell killing (27). The truncated form of DT used in this study (DT₃₉₀) was selected due to previous research describing a series of internal frame deletion mutations that established amino acid 389 as the best location for genetic fusion of DT to targeting ligands (28). DT₃₉₀ contains the A fragment of native DT that catalyzes ADP ribosylation of elongation factor 2 (EF-2) leading to irreversible inhibition of protein synthesis and cell death (29,30).

This study set out to address the hypothesis that including two well-established targeting ligands on the same single chain molecule would have distinct targeting advantages over the same monospecific targeting agents. Thus, a new bispecific CT, DTEGF13, was bioengineered and compared to the monospecific cytotoxins DTEGF and DTIL13. Agents were tested against several aggressive human pancreatic tumor cell lines in vitro and against MiaPaCa-2 in vivo which are readily xenografted into athymic nude mice.

Materials and Methods

DTEGF13 Construction

DNA shuffling and PCR assembly techniques were used to assemble the genes encoding the single chain bispecific CT DTEGF13. From the 5' end to 3' end, the assembled gene consisted of an *NcoI* restriction site, an ATG initiation codon, the first 389 amino acids of the DT molecule (DT₃₉₀), the 7 amino acid EASGGPE linker, the genes for human EGF and IL-13 linked by a 20 amino acid segment of human muscle aldolase (hma), and a *XhoI* restriction site (Figure 1A). The primers used for the final assembly of DTEGF13 are shown in Table 1. The final 1755bp *NcoI/XhoI* target gene was spliced into the pET21d expression vector under control of an isopropyl-b-D-thiogalactopyranoside (IPTG) inducible T7 promoter. The gene was correct in sequence and was been cloned (Biomedical Genomics Center, University of Minnesota). The monospecific agents, DTEGF and DTIL13 were created using the same techniques.

A pair of bivalent immunotoxins targeting hematological malignancies were produced and used as specificity controls in this study. DT2222, a bivalent fusion toxin control containing the same DT₃₉₀ cassette, was produced by joining two repeating scFvs specific for human CD22 to DT₃₉₀. CD22 is human B lymphocyte-specific glycoprotein that is expressed in the majority of B-cell leukemias and lymphomas (31). Bic3 is a T-cell specific immunotoxin consisting of two consecutive scFvs recognizing the human CD3ε linked to DT₃₉₀ (32).

Isolation of inclusion bodies, refolding and purification

These procedures were previously described (32). Plasmids were transformed into *Escherichia coli* strain BL21(DE3) (Novagen, Madison WI). Following overnight culture, bacteria were grown in Luria broth. Gene expression was induced with the addition of IPTG (FischerBiotech, Fair Lawn, NJ). Two hours after induction, bacteria were harvested by centrifugation. Cell pellets were suspended and homogenized. Following sonication and centrifugation, the pellets were extracted and washed. Inclusion bodies were dissolved and protein refolded. Refolded proteins were purified by fast protein liquid chromatography ion exchange chromatography (Q sepharose Fast Flow, Sigma) using a continuous gradient.

Tissue Culture

The human pancreatic cell lines MiaPaCa-2 and PANC-1, and the Burkitt's Lymphoma cell line Daudi (33) were obtained from the American Type Culture Collection (ATCC, Rockville MD). The H2981-T3 human lung adenocarcinoma line has been described previously (34). Cells were maintained in DMEM (MiaPaCa-2 and PANC-1) or RPMI-1640 (H2981-T3 and Daudi) media (Cambrex, East Rutherford NJ) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. All carcinoma cells were grown as monolayers and Daudi cells in suspension using culture flasks. Cell cultures were incubated in a humidified 37°C atmosphere containing 5% CO₂. When adherent cells were 80–90% confluent they were passaged using trypsin-EDTA for detachment. Only cells with viability >95%, as determined by trypan blue exclusion, were used for experiments.

Measuring cell kill by proliferation inhibition

To determine the effect of DTEGF13 on pancreatic cancer cells, proliferation assays measuring ³H-thymidine incorporation were performed (32). Cells (10⁴/well) were plated out in a 96-well flat-bottomed plate and incubated overnight at 37°C with 5% CO₂ to allow cells to adhere. Cytotoxins in varying concentrations were added to wells in triplicate. Incubation at 37°C and 5% CO₂ continued for 72 hours. [Methyl-³H]-thymidine (GE Healthcare, UK) was added (1 µCi per well) for the final 8 hours of incubation. Plates were frozen to detach

cells and cells were then harvested onto a glass fiber filter, washed, dried, and counted using standard scintillation methods. Data from proliferation assays are reported as percentage of control counts. For blocking studies, increasing concentrations of EGF13 were mixed with 1 nM DTEGF13 and subsequent mixtures were added to wells containing MiaPaCa-2 cells. All other aspects of blocking assays were identical to the procedure listed above for proliferation assays.

In vivo efficacy studies

Male *nu/nu* mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center, Animal Production Area and housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited specific pathogen-free facility under the care of the Department of Research Animal Resources, University of Minnesota. Animal research protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. All animals were housed in microisolator cages to minimize the potential of contaminating virus transmission. Three different flank tumor studies were performed for this study. In each of the experiments tumor volume was determined as a product of $W \times L \times H_{[sbl2]}$ as measured by calipers. Animal weights were also monitored as an indication of treatment-related toxicity. Mice with flank tumors $>2.0 \text{ cm}^3$ were euthanized in accordance with University of Minnesota Research Animal Resources guidelines.

Experiment 1—Flank tumors were initiated by injecting 1×10^7 MiaPaCa-2 cells in DMEM into the left flank of nude mice ($n=20$). On day 22, mice with tumors exceeding 50 mm^3 in volume were randomized into DTEGF13 treatment or no treatment groups. Treatment mice received $2.5 \mu\text{g}$ DTEGF13 in $100 \mu\text{l}$ PBS injected intratumorally. A total of 10 injections were given on days 22, 24, 27, 29, 31, 36, 38, 42, 45, and 48 (Figure 5B).

Experiment 2—One day prior to cell injection, male nude mice were irradiated with 3 Gy using an X-ray irradiator. Flank tumors were established by injecting 1×10^7 MiaPaCa-2 cells in a 1:1 mixture of DMEM and Matrigel (BD Biosciences, San Jose CA). When tumors reached approximately 50 mm^3 (day 18), mice were divided into groups ($n=5/\text{group}$) and treatment was initiated. Four injections of $2.5 \mu\text{g}$ DTEGF13, DTEGF, or DTIL13 were given intratumorally QOD on days 18, 20, 22, and 24. Mice in the control group received intratumoral injections of $100 \mu\text{l}$ PBS.

Experiment 3—For the final flank tumor study, nude mice were injected with 1×10^7 MiaPaCa-2 cells in a 1:1 mixture of DMEM and Matrigel. On day 15 when flank tumors were approximately 75 mm^3 , mice were divided into treatment groups ($n=6/\text{group}$). Treatment mice received a total of 6 intratumoral injections of $2.5 \mu\text{g}$ of either DTEGF13 or the B-cell targeting immunotoxin DT2222. Injections were given on days 15, 17, 19, 23, 25, and 28.

Statistical analyses

All statistical analysis was performed using Prism 4 (Graphpad Software, Sand Diego CA). Groupwise comparisons of single data points were made by Student's t-test. Probability (p) values < 0.05 were considered significant.

Results

Purification of DTEGF13

Following refolding and purification, batches of DTEGF13 were analyzed by SDS-PAGE (Figure 1B) and size exclusion chromatography. Both analyses revealed that DTEGF13 was the expected molecular weight (63.6 kDa) and purity was $\geq 95\%$. Final yields of purified protein were 5–10 mg/L of culture.

The ability of DTEGF13 to inhibit proliferation of pancreatic tumor cells

To determine the ability of DTEGF13 to kill EGFR-expressing and IL-13R-expressing pancreatic cancer cells, it was tested against the EGFR⁺ and IL-13R⁺ PANC-1 tumor cell line (Figure 2). Bispecific DTEGF13 showed an IC₅₀ (concentration that inhibits 50% of cell proliferation) of 0.035 nM indicating that it was at least 2,800-fold more toxic than either DTEGF or DTIL13 which did not reach an IC₅₀. Bic3, a T-cell targeting IT containing the DT₃₉₀ molecule was included as a specificity control. DTEGF13 also showed potent cytotoxicity against the SW-1990 and ASPC-1 pancreatic cancer cell lines with an IC₅₀ of 0.00013 and 0.052 nM respectively (not shown). Killing in proliferation assays was verified by trypan blue staining (not shown).

Increased activity of DTEGF13 is due the presence of EGF and IL-13 ligands on a single molecule

Proliferation assays were conducted in order to determine if the increased activity of DTEGF13 was a result of the increased number of binding molecules present on a bispecific CT. Figure 3 shows the data comparing the activity DTEGF13 to the monospecific DTEGF and DTIL13, as well as a combination of both monospecific CT against MiaPaCa-2 pancreatic (Fig 3A) and H2981-T3 lung (Fig 3B) cancer cells. A mixture of DTEGF and DTIL13 results in an identical number of ligands as are present in the same concentration of DTEGF13. Against MiaPaCa-2 cells the monospecific DTEGF was able to kill with an IC₅₀ of 0.007 nM. Monospecific DTIL13 was less effective with an IC₅₀ of 30 nM. However, DTEGF13 showed an IC₅₀ of 0.00017 nM, representing a 41-fold increase in activity as compared to DTEGF and a 176,000-fold increase in activity as compared to DTIL13. Interestingly, a mixture of DTEGF and DTIL13 showed no increased in activity over DTEGF alone. DTEGF13 also demonstrated broad reactivity with a number of EGFR⁺ and IL-13R⁺ carcinoma cell lines, including the human lung cancer cell line H2981-T3 (Figure 3B). As in the case of MiaPaCa-2, DTEGF13 was the most effective with an IC₅₀ of 0.002 nM. DTEGF showed an IC₅₀ of 0.25 nM and DTIL13 showed an IC₅₀ of 100 nM, an increase of 125-fold and 50,000, respectively. Once again, a combination of DTEGF and DTIL13 showed no advantage in cytotoxicity over either monospecific CT. These data demonstrate the superior activity of DTEGF13 is due to the presence of both ligands on a single-chain molecule.

Specificity of DTEGF13 cytotoxicity

In order to test the specificity of DTEGF13 activity, two different assays were conducted. Figure 4A shows that neither DTEGF13, nor the monospecific DTIL13 or DTEGF CT had any effect on the proliferation of EGFR⁻ and IL-13R⁻ Daudi lymphoma cells. In Figure 4B we demonstrate that the activity of 1 nM DTEGF13 can be blocked by adding saturating concentrations of EGF13 to cultures of MiaPaCa-2 cells. EGF13 is a recombinant molecule identical to DTEGF13 except that it lacks the DT₃₉₀ fragment. A 10-fold excess of EGF13 was able to inhibit more than 80% of DTEGF13-mediated cell killing. Higher concentrations of EGF13 fully abrogated CT activity. The specificity of blocking was confirmed by the fact that even a 1000-fold excess of 2219EA, a B-cell targeting bispecific protein, was unable to affect the activity of DTEGF13. These data demonstrate the highly specific nature of DTEGF13 cytotoxicity.

Efficacy of intratumoral DTEGF13 in a MiaPaCa-2 nude mouse flank tumor model

To test the ability of DTEGF13 to inhibit pancreatic tumor growth in vivo, human MiaPaCa-2 cells were xenografted into the flank of nude mice. Once the tumors were established and palpable, mice were treated with multiple intratumoral injections. For Experiment 1 shown in Figure 5, 1×10⁷ tumor cells suspended in DMEM were injected into mice that had not been subjected to total body irradiation (TBI). This method yielded a poor tumor establishment rate

(<40% of injected animals). Three of the animals that did form tumors were given an aggressive course of 10 injections of DTEGF13 over the course of three weeks, as established from earlier pilot experiments. In this experiment, control tumor growth was slower than desired in most animals (Figure 5A). However, Figure 5B shows that the course of DTEGF13 treatment appeared to keep tumor growth in check over the duration of the short study.

In Experiment 2 (Figure 6), animals were given 3 Gy TBI one day prior to the injection of 1×10^7 MiaPaCa-2 cells. Cells were injected in a 1:1 mixture of DMEM and Matrigel in order to promote better tumor growth. The combination of TBI and Matrigel increased the tumor take rate to >95%. When treatment was initiated (day 18), mice in the treatment groups received 4 intratumoral injections of 2.5 μ g of either DTEGF13, DTEGF, or DTIL13 given every other day. Control mice received intratumoral injections of PBS on the same schedule. Figure 6A shows that the highest degree of anti-tumor efficacy was achieved with DTEGF13 administration. Figure 6B shows the tumor volumes of the individual animals in the DTEGF13 treatment group. Each of the animals showed a noticeable decrease in tumor volume with one tumor completely regressing. However, Figure 6C shows that treatment-related toxicity was heightened by the total body irradiation given to the animals in this experiment. Weight loss and mortality (3/5 animals) occurred despite following a previously well-tolerated treatment regimen. Others have reported that immunotoxin toxicity can be exacerbated by irradiation (33).

For Experiment 3 (Figure 7), unirradiated mice were injected subcutaneously in the left flank with 1×10^7 MiaPaCa-2 cells suspended in 100 μ l of a 1:1 mixture of DMEM and Matrigel. This method facilitated 100% tumor establishment without introducing the unwanted side effects related to TBI. Figure 7A shows significant anti-tumor effect of DTEGF13 compared to the tumor progression observed in groups of mice that were untreated or were treated with the negative control DT2222. Tumor growth was contained, but relapses did occur following cessation of DTEGF13 treatment. A course of 6 intratumoral injections of 2.5 μ g DTEGF13 was tolerated with no significant toxicity as evidenced by animal weight (Figure 7B).

Together, these studies show that in a model in which the human EGF and IL-13 of DTEGF13 is cross-reactive with mouse EGFR and IL-13R, DTEGF13 is a highly effective anti-tumor agent. The agent is highly selective in its action against pancreatic cancer and both EGF and IL-13 moieties positioned on the same molecule are necessary for its superior anti-tumor effect.

Discussion

The original contribution of this study is that IL-13 and EGF were cloned into the same single chain molecule with truncated diphtheria toxin. The new recombinant hybrid anti-pancreatic cancer agent had far greater activity than CT made with either cytokine alone. This new DTEGF13 was potent and highly effective against MiaPaCa-2 tumor cells in vitro displaying an IC_{50} of 0.00017 nM, representing a 41-fold increase in activity over DTEGF and a 176,000-fold increase in activity over DTIL-13. Studies with mixtures of monomeric CT showed that the enhanced activity of DTEGF13 was dependent on having both ligands on the same single chain molecule. Additionally, DTEGF13 had striking effects against pancreatic tumors in vivo. Despite the aggressive nature of the Mia-PaCa-2 flank tumor in xenografted nude mice, all of the tumors responded including one tumor that did not reoccur after treatment. Another important aspect of this study is that EGF and IL-13 are cross-reactive with mouse EGFR and IL-13R in this mouse model (constituting an “on target” model). Multiple injections of DTEGF13 were tolerated. Thus, a therapeutic index clearly exists.

Others have investigated the potential of using IL-13R and EGFR-targeted toxins for therapy of this aggressive pancreatic cancer. For example, investigators have enhanced IL-13R α 2 gene expression in pancreatic cancer cells and showed that these IL-13R expressed tumors had

susceptibility to IL13 CT (11). Interestingly, these investigators reported a dominant infiltration of cells including macrophages and natural killer cells in the regressing tumors. Since macrophages were found to produce nitric oxide, IL-13R α 2-targeted cancer therapy involved not only a direct tumor cell killing by IL-13 cytotoxin but also activation of innate immune response at the tumor site. It will be important to determine whether DTEGF13 is activating the immune response. This study also points out that IL-13 CT are not effective against all pancreatic tumors, probably because they differ in the amount of surface IL-13R expressed. Since DTEGF13 appears to require limited IL-13R expression to enhance tumor kill, it is a very attractive anti-pancreatic cancer agent.

For EGF, studies show a recombinant immunotoxin made by genetically fusing the anti-EGFR single chain variable fragment to truncated *Pseudomonas aeruginosa* exotoxin A showed specific binding to and toxicity against the EGFR-positive, metastatic pancreatic carcinoma cell line L3.6pl, but not to control cell systems (6,7). Both single and multiple injection treatment protocols resulted in a significant reduction in the average number of lung metastases in tumor bearing animals indicating that targeting the EGFR with CT is an effective strategy against disseminated human pancreatic carcinoma cells. Together, these studies suggest that targeting EGFR and IL13R simultaneously may have clear advantages.

In the *in vivo* experiments performed in this paper, experiment 1 showed that DTEGF13 was effective, but control tumor growth was slow. In experiment 2, DTEGF13 was effective again, but toxicity was enhanced because total body irradiation was given to enhance tumor growth. Others have shown in human lymphoma models that irradiation can enhance immunotoxin related side effects (36,37). This appears to be also true of DTEGF13. Others are exploring the efficacy of targeting EGFR with radiolabeled antibodies (38,39). Combining this form of therapy with our DTEGF13 CT therapy would be unadvisable without thorough investigation. However, other forms of combined therapy may be very effective. For example, phase 1 studies are currently examining combining anti-EGFR antibody therapy with gemcitabine chemotherapy (40). Gemcitabine is the most successful form of chemotherapy for pancreatic cancer. Because CT have a mechanism of killing that is entirely different from the mechanism of kill of chemotherapy, many studies have shown that chemotherapy can be successfully combined with immunotoxin or cytotoxin therapy (41–43). Thus, the probability is high the effectiveness of DTEGF13 therapy can be heightened by combining it with chemotherapy. In fact, investigators have combined IL-4 CT with gemcitabine heightening anti-pancreatic cancer efficacy (44). In fact, our preliminary studies with MiaPaca-2 show that adding 50 nM gemcitabine reduces the IC₅₀ of DTEGF13 over 3 logs *in vitro*. In experiment 3, we successfully enhanced tumor growth by injecting cells in Matrigel and DTEGF13 was highly effective at inhibiting flank tumor growth.

The combined effect of the monomeric DTEGF and DTIL13 CT was not greater than the individual effects of the monomeric CT, but when IL-13R and EGFR were targeted simultaneously by linking both cytokines on the same molecule, the anti-cancer effect was enhanced. The reason could potentially relate to enhanced receptor numbers, enhanced internalization, or differences in intracellular, subcellular compartmentalization. Studies with prostate cancer cells show that enhanced activity of DTEGF13 may not be solely attributed to binding (45). Past studies show that immunotoxin activity relates to compartmentalization within the cell. Studies of the interrelationships of the IL-13R and EGFR pathways in a primary normal human bronchial epithelial cell culture system via microarray analysis indicate that the two pathways have independent effects (39). The enhanced activity of DTEGF13 could relate to improved internalization whereby the toxin is delivered more efficiently to the cytosol, the main target organelle of catalytic toxins. The ligands themselves are not contributing to the cytotoxicity, since we synthesized EGF13 devoid of toxin and this molecule did not inhibit proliferation of cells that were killed by DTEGF13.

As discussed, toxicity issues will need to be addressed more thoroughly. Mice tolerated DTEGF13 very well at the dose of 2.5 ug/injection (100 ug/kg). However, tolerability was dependent on spacing the doses. Intratumoral injection is not an effective means of delivery since areas of the tumor are often not injected and relapses occur in these neglected tumor areas. Moreover, injection precipitously spikes the dosage creating steep peaks and rapid troughs. Studies by others indicate that pump delivery may be superior providing constant dose delivery over a far longer period of time.

Other bispecific fusion toxins have been reported. In some reports, inclusion of a second ligand enhances activity (35,46), but not in others (47). Currently, no method exists which predicts whether a given bispecific will be successful.

In conclusion, DTEGF13 represents a powerful new anti-pancreatic cancer agent. Its construction is based on molecules that react with well-studied and established cancer targets, IL13R and EGFR. In vitro studies show conclusive proof that the presence of both ligands on the same molecule is responsible for its superior activity. Animal studies in a model in which human DTEGF13 is cross-reactive with the native mouse receptors indicate that it is highly effective in checking aggressive tumor progression and reasonably tolerated. DTEGF13 may be a useful alternative therapy for pancreatic cancer and perhaps other carcinomas.

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Abbreviations

CT, cytotoxin; DT, diphtheria toxin; EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; IC₅₀, Inhibition concentration 50%; IL-13, Interleukin 13; IL13R, Interleukin 13 receptor; IL4R, Interleukin 4 receptor.

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Figure 1. Construction and purification of DTEGF13

A) The gene fragment encoding the single-chain bispecific immunotoxin DTEGF13 was created using overlap extension PCR. This construct consisted of: (from 5' to 3') a truncated diphtheria toxin molecule (DT₃₉₀), a seven amino acid (EASPPGE) linker, human epidermal growth factor (EGF), a flexible 20 amino acid segment of human muscle aldolase (hma), and interleukin-13 (IL-13). Using the *NcoI/XhoI* restriction sites the sequence for DTEGF13 was cloned in the pET21d bacterial expression vector. **B)** SDS-PAGE analysis was used to analyze the expression and purification of DTEGF13. Lane 1-Inclusion bodies isolated from *E. coli* following IPTG-induction of DTEGF13 expression. Lane 2-Molecular weight standards. Lane 3-DTEGF13 (63.6 kDa) protein following in vitro refolding and purification.

Figure 2. Potent cytotoxicity of bispecific cytotoxin DTEGF13

The in vitro activity of bispecific DTEGF13 and monospecific DTEGF and DTIL13 was determined by measuring 3H -thymidine incorporation into PANC-1 cells following 72 hour incubation with varying concentrations of CT. BIC3, a T-cell targeting immunotoxin containing DT₃₉₀ was included as a negative control. Data is expressed as percentage of 3H -thymidine relative to control cells incubated in media alone (Control counts=30290±5091). Points represent mean of triplicate measures±SD. IC_{50} indicates the concentration of CT that inhibits 50% of cell proliferation relative to untreated cells.

Figure 3. Enhanced cytotoxicity of bispecific DTEGF13 is dependant on linking both EGF and IL-13 on a single-chain molecule

The anti-proliferative effect of DTEGF13, DTEGF, DTIL13, and a mixture of DTEGF/DTIL13 on **A)** MiaPaCa-2 (pancreatic) and **B)** H2981-T3 (lung) cancer cells was tested by measuring 3H -thymidine uptake 72 hours following CT exposure. Points on each graph represent mean of triplicate samples $\pm$ SD. Control counts= A) 72048 $\pm$ 9826, B) 64876 $\pm$ 11866.

Figure 4. Specificity of DTEGF13

A) EGFR⁻ and IL-13R⁻ Daudi cells were incubated with DTEGF13, DTEGF, and DTIL13. **B)** DTEGF13 mediated cytotoxicity was inhibited by adding increasing concentrations of bispecific EGF13, which lacks a DT₃₉₀ moiety, to cultures of MiaPaCa-2 cells incubated with 1 nM DTEGF13. 2219EA, a bispecific B-cell targeting molecule was used as an irrelevant negative control. In both experiments cell proliferation was determined by measuring ³H-thymidine incorporation following 72 hours incubation time. Data is expressed as percentage of cell-associated ³H-thymidine at each concentration relative to control cells. Points represent means±SD.

Experiment 1 - MiaPaCa-2 - No Matrigel

Figure 5. Effect of intratumoral injection of DTEGF13 on MiaPaCa-2 flank tumors-Experiment 1 MiaPaCa-2 flank tumors were established by injecting 1×10^7 cells into the left flank of male nude mice. Once palpable tumors were established (day 22) mice were divided into two groups, **A)** No treatment or **B)** DTEGF13 treatment. Mice in the DTEGF13 group received 2.5 µg DTEGF13 injected intratumorally as often as indicated by arrows on graph.

Figure 6. Effect of intratumoral injection of DTEGF13 on MiaPaCa-2 flank tumors-Experiment 2 Prior to injection of tumor cells, male nude mice were irradiated with 3 Gy using an x-ray irradiator. Flank tumors were then established by injecting 1×10^7 MiaPaCa-2 cells in a 1:1 mixture of DMEM:Matrigel. When tumors reached approximately 50 mm³, mice were divided into groups and treated with intratumoral injections of 2.5 µg DTEGF13, DTIL13, DTEGF, or PBS. Four injections were given QOD as indicated by arrows on graph. **A)** Average tumor volume of animals in each treatment group. **B)** Individual tumor volumes and **C)** Animal weights of individual mice in the DTEGF13 treatment group.

Experiment 3 - MiaPaca-2 No Irradiation, Matrigel

Figure 7. Effect of intratumoral administration of DTEGF13 on MiaPaCa-2 flank tumors-Experiment 3

A) A final xenograft model of pancreatic cancer was established by injecting male nude mice (no prior irradiation) with 1×10^7 MiaPaCa-2 cells in a 1:1 mixture of DMEM:Matrigel. Mice were randomized into three groups (n=6/group) on day 15 when the average tumor volume was approximately 75 mm³. Treated animals were injected intratumorally with 2.5 μ g of either DTEGF13 or the B-cell lymphoma targeting DT2222. A total of 6 injections were given over the course of two weeks as indicated by arrows on graph. **B)** Weights of animals in the DTEGF13 treatment group.

Table 1

Sequence of oligonucleotides used to assemble DTEGF13.

Primer	Characteristics	Sequence
A	the sense primer introduced an NcoI restriction site (underlined) with an initiation codon ATG and the first 7 codons of DT	5' CAG <u>cc</u> ATGGGCGCTGAT GATGTTGTTGAT 3'
B	the antisense primer introduced the codons 383 to 389 of DT and codons of the linker EASGGPE	5' ctgggacacctcggagcttcAAATG GTTGCGTTTTATGCCC 3'
C	the sense primer introduced the codons of EASGGPE linker and codons 1 to 7 of human EGF	5' GAAGCTTCCGGAGGTCCC GAGaagcagcgacgagaatgtccg 3'
D	the antisense primer introduced last 7 codons of human EGF and the codons of Part of the HMA linker with NheI restriction site (underlined)	5' TTC <u>gct</u> AGCAGCAGCACC AGCCTGACCAGACGGgcgc agttccaccatttcag 3'
E	the sense primer introduced part of the HMA linker with NheI restriction site (underlined) and codons 1 to 7 of human IL13	5' GCTGCT <u>agc</u> GAATCTCTGT TCGTTTCTAACCACG3' CTTACggccctgtgcctccctctaca3'
F	the antisense primer introduced the last 7 codons of human IL13 and XhoI restriction site (underlined) with stop codon in front.	5' cagCTCGAGCTAgtgaaccgt ccctcgcgaaa 3'