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A new drug delivery method of bispecific ligand-directed toxins that reduces toxicity and promotes efficacy in a model of orthotopic pancreatic cancer

Seunguk Oh, PhD,

Department of Therapeutic Radiology, University of Minnesota Cancer Center, Minneapolis, Minnesota

Brad J Stish, MS,

Department of Therapeutic Radiology, University of Minnesota Cancer Center, Minneapolis, Minnesota

Selwyn M. Vickers, MD,

Department of Surgery, University of Minnesota Cancer Center, Minneapolis, Minnesota

Donald J. Buchsbaum, PhD, and

Department of Radiation Oncology, University of Alabama at Birmingham

Daniel A. Vallera, PhD

Department of Therapeutic Radiology, University of Minnesota Cancer Center, Minneapolis, Minnesota

Abstract

Objective—Biologicals targeting EGFR and IL-13R react with over-expressed markers on cancer cells, but also react with receptor on normal cells. Since we developed novel bispecific ligand directed toxins (BLT) synthesized by cloning EGF and IL-13 on the same molecule with toxin, our objective was to determine whether we could block normal receptors while still targeting receptors over-expressed on cancer cells thereby decreasing toxicity while maintaining efficacy.

Methods—A method, ToxBloc, was developed in which a bolus IP dose of recombinant EGF13 (without toxin) was given to mice about 15-20 minutes prior to DTEGF13. Experiments were then performed to determine if the MTD was reduced and whether we still were able to eliminate progression of aggressive human, metastatic, systemic pancreatic cancer induced by orthotopic injection in nude mice.

Results—ToxBloc permitted us to safely exceed the DTEGF13 MTD by 15-fold. This approach permitted repetitive high dosing with the BLT resulting in tumor regression ($p < 0.01$). Tumor affects were documented using a tumor imaging model in which tumor growth was monitored noninvasively in real time. ToxBloc was selective since other bispecific peptides did not block.

Conclusions—ToxBloc represents a new method of drug delivery and a potential solution to the toxicity problem.

Corresponding author DAV: MMC:367 Minneapolis, MN 55455 valle001@umn.edu, phone 612-626-6664, fax 612-624-3913.

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Keywords

immunotoxin; pancreatic cancer; nude mice; EGF; IL-13; toxicity

Introduction

Researchers have expended great efforts to identify new drug delivery methods in hopes of identifying the best method to direct the drug to tumor while avoiding critical non-target organs [1]. Biologicals, particularly targeted toxins, have been limited by their toxicity so that the identification of a new method, particularly one that is simple, easily employed, and not necessarily device dependent would be highly desirable [2].

Targeted catalytic toxins are still under investigation as potential anti-cancer drugs because they are perhaps the most potent anti-cancer drugs, killing tumor cells in picomolar concentrations. Their toxic side effects are a chief limitation. We have bioengineered unique bispecific ligand directed toxins (BLT) that show clear advantages over their monospecific counterparts [3-5]. One of these, called DTEGF13, was created by linking the catalytic fragment of diphtheria toxin (DT) to human EGF and IL-13 [6,7]. Both EGFR and IL13R are overexpressed on pancreatic cancer cells and expressed on some normal tissues [8,9]. DT₃₉₀ was selected due to previous research describing a series of internal frame deletion mutations, establishing amino acid 389 as the best location for genetic fusion of the toxin to targeting ligands [10]. The A fragment of DT₃₉₀ is known to catalyze ADP ribosylation of elongation factor 2 (EF-2) leading to irreversible inhibition of protein synthesis and cell death [11,12].

Pancreatic adenocarcinoma is highly aggressive and only a small percentage (10-15%) of cases present with resectable tumor at diagnosis [13]. Due to its highly aggressive nature and rapid metastasis, at least 32,000 patients die yearly of this cancer in the U.S. alone [14]. Studies have shown that chemotherapy can extend survival, but the survival benefit is less than six months [15,16]. Because of the high level of EGFR and IL13R overexpression, human pancreatic cancer cells make excellent targets for toxin targeting and hopes for an effective alternative drug are high [17-19].

Genetic engineering has afforded us the opportunity to devise a new drug delivery strategy contrasting sharply to various device methods such as pumps and liposomes that have shown limited success with biologicals. The method called toxicity blocking (ToxBloc) was predicated on the understanding that most over-expressed receptors targeted by biologicals on cancer cells are also expressed, albeit to a lesser degree, on critical non-target organ cells. The strategy consists of administering a large bolus IP dose of genetically engineered EGF13 (devoid of toxin) to mice prior to a high, lethal dose of DTEGF13 also given IP. Several years ago studies with radiolabeled conventional antibodies showed that administration of unlabeled antibody prior to the same radiolabeled antibody could improve tumor localization of radiolabeled antibody [20]. Although no data indicated that survival was improved, the data supports the hypothesis that normal receptors can be blocked with a positive outcome.

This study set out to determine if ToxBloc can extend the MTD resulting in a subsequent and significant anti-cancer effect against a highly aggressive, malignant orthotopic pancreatic cancer xenograft model.

Materials and Methods

DTEGF13 Construction

DNA shuffling and PCR assembly techniques were used to assemble the genes encoding DTEGF13 and EGF13. For 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 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. DNA analysis was used to verify that the gene was in correct sequence (Biomedical Genomics Center, University of Minnesota). Bic3 was synthesized as a control by fusing two repeating scFvs recognizing human CD3epsilon to DT₃₉₀.

For the ToxBloc studies, we also bioengineered a DNA fragment containing only the two ligands EGF13 (Mw. 24471), the same EGF13 used in the synthesis of DTEGF13. As specificity controls, we also synthesized 2219 (Mw. 57152), the V_H and V_L regions of anti-CD22 (scFv) and anti-CD19 scFv spliced together on a single chain molecule [4] and EGF4 (Mw.24087), the human EGF cytokine spliced to human IL-4.

Isolation of inclusion bodies, refolding and purification

These procedures were previously described [7]. 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-Aldrich, St. Louis, MO) using a continuous gradient.

Cell Lines

The human pancreatic cell line MiaPaCa-2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). MiaPaCa-2 was stably transfected with vector containing both the firefly luciferase (Luc) and green fluorescent protein (GFP) genes, as well as a blastocidin resistance gene (Clontech Laboratories, Mountain View California, USA). Transfection was performed with Lipofectamine reagent (Invitrogen, Carlsbad California, USA) and stable clones were established using a FACSDiva flow cytometer (University of Minnesota Flow Cytometry Core Facility of the Masonic Cancer Center) to seed individual GFP-positive cells into a 96-well plate. Each clone retained identical morphological and biological properties to the specific parental cell line. Cells were maintained in DMEM media (Cambrex, East Rutherford, NJ) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 ug/mL streptomycin. MiaPaCa-2-luc cells were maintained with additional 10 ug of Blastocidin (InvivoGen, San Diego, CA). Cell cultures were incubated in a humidified 37°C atmosphere containing 5% CO₂. When 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 in vitro

To determine the effect of DTEGF13 (Mw. 63785) on pancreatic cancer cells, proliferation assays measuring ³H-thymidine incorporation were performed [21]. Cells (10⁴/well) were

plated into a 96-well flat-bottomed plate and incubated overnight at 37°C with 5% CO₂ for adherence. Cytotoxins in varying concentrations were added to wells in triplicate. Incubation continued for 48 hours. [Methyl-³H]-thymidine (GE Healthcare, UK) was added (1 uCi 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 untreated 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.

Flow Cytometry Analysis of DTEGF13 binding

In order to measure binding to MiaPaCa-2 cells, DTEGF13 was labeled with fluorescein isothiocyanate (FITC) as described previously [7]. FITC isomer was conjugated to DTEGF13 at a 6:1 molar ratio using a 0.5M borate buffer, pH 9.0. After 3hrs at room temperature on a rotating platform, unbound FITC was separated from bound using a NAP-5 column (GE Healthcare, UK). FITC-labeled DTEGF13 at increasing concentrations was incubated with 10⁶ cells on ice for 30 minutes to allow binding. Following incubation cells were washed three times. Binding was measured from triplicate samples using FACS Calibur and CellQuest software (BD Biosciences, San Jose CA). Mean fluorescence index (MFI) of DTEGF13 binding was determined by subtracting the mean fluorescence of cells incubated with control AHN-12 from that of cells incubated with DTEGF13. The K_d was calculated by GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) using non-linear regression analysis. The technique and its use are discussed in the Prism manual entitled Analyzing Data with GraphPad Prism.

Orthotopic Tumor Model and Real Time Imaging

Male nu/nu mice or normal 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 horizontal pathogenic contamination.

For orthotopic inoculation of MiaPaCa-2-luc cells, mice were anesthetized with 1.6ul/g of ketamine/xylazine cocktail (ketamine 6.5 mg/ml, xylazine 0.44 mg/ml). A small 0.5 cm incision was made and 4 × 10⁶ cells were injected orthotopically into the exposed pancreas (1:1 mixture cell:matrigel) (BD bioscience, San Jose, CA) using a 26 gauge needle and a Hamilton syringe (see Figure 3). In some experiments, dye was injected with cells in extra mice to confirm that cells were not leaking from the injection site. The peritoneum was then closed with dissolvable suture and the skin incision closed with wound clips (Stoelting Co, Wood Dale, IL).

The effect of BLT DTEGF13 against the MiaPaCa-2-luc orthotopic tumors was measured using bioluminescent imaging in real time. Mice were imaged every week to monitor the level of luciferase activity and tumor progression. Images were captured using Xenogen Ivis 100 imaging system and analyzed with Living Image 2.5 software (Xenogen Corporation, Hopkington, MA). During imaging, mice were lightly anesthetized with isoflurane gas. All mice received 100 µl of a 30 mg/ml D-luciferin aqueous solution (Gold Biotechnology, St. Louis MO) IP 10 minutes before imaging to provide a substrate for the luciferase enzyme. All images represent 5 minutes exposure time and all regions of interest (ROI) are expressed

in units of photons/sec/cm²/sr. For fluorescent imaging, images were recorded using Maestro Imaging Station (CRI, Woburn MA).

Toxicity Blocking (ToxBloc) Reduces Toxicity and Elevates the Tolerated Dose

For toxicity blocking, bioengineered EGF13 was given to groups of mice with pancreatic tumors. A dose of 200 ug EGF13 in 500 ul low endotoxin PBS administered IP was followed 15-20 minutes later with a lethal dose of 7.5 ug DTEGF13 in 100 ul PBS. Treatment schedule was injection/every other day (three times/week, MWF). This was called one "course of treatment". The 7.5 ug DTEGF dose was about 15-fold higher than the MTD and exceeds a lethal dose by 8-10-fold.

To find optimum interval between blocking peptide EGF13 and BLT DTEGF13, mice (N=3) were treated at 5 and 30 minutes intervals between the 200 ug EGF13 dose and the 7.5 ug DTEGF13 dose. In all experiments, animals were injected with one full MWF course and body weight, appearance and behavior of each animal was monitored. Organs were removed from extra animals given either ToxBloc or 200 ug DTEGF13 without the EGF13 block the day after the second injection. Liver tissue and tissue from other organs including heart, lung, kidney and gut were embedded in OCT compound (Sakura, Tokyo), snap frozen in liquid nitrogen, and stored at -80° C until sectioned. Serial 4 µm sections were cut, thaw mounted onto glass slides, and fixed for 5 minutes in acetone. Slides were stained with hematoxylin and eosin (H&E) for histopathological assessment.

Efficacy of ToxBloc treatment

To determine the ability of ToxBloc to inhibit the progression of systemic tumors implanted OT, four experiments were performed.

In experiment 1, athymic nude mice were injected orthotopically (OT) with 4 million MiaPaCa2-luc cells on day 0 (N=5/group). Animals were imaged and then received a single course of ToxBloc treatment on days 7, 9, 11. A course was defined as 3 injections/week (MWF).

In experiment 2, multiple course treatment with ToxBloc was tested. Athymic nude mice were injected OT with 4 million Mia PaCa2-luc cell on day 0 (N=4-5/group). Five courses of ToxBloc treatment totaling 15 injections were administered starting days 4, 11, 18, 25, and 32 post-tumor injection.

In experiment 3, ToxBloc was tested with a lower tumor burden, 2 million Mia PaCa2-luc cells injected OT. Groups included ToxBloc, and no treatment. Five courses of ToxBloc treatment totaling 15 injections were given starting on days 10, 21, 44, 62, and 70.

In Experiment 4, the ToxBloc study was performed with larger size animal groups (n=7/group). Mice were injected OT with 4 million MiaPaCa2-luc cell on day 0. Five courses of ToxBloc treatment totaling 15 injections were given on days 5, 12, 19, 26, and 33.

Statistical analyses

All statistical analyses of in vivo data were performed using Prism 4 (Graphpad Inc, San Diego CA). Groupwise comparisons of mean data were made by Student's t-test. Probability (p) values < 0.05 were considered significant.

Results

DTEGF13

Following purification, DTEGF13 was the expected molecular weight (63.6 kDa) with a purity of 92% and EGF13 was the expected molecular weight (24.5 kDa) with a purity of 95% (not shown). To determine the potency of DTEGF13 proliferation studies were performed on Mia PaCa2 cells (Figure 1B). DTEGF13 was the most potent with an IC_{50} of 0.0009 nM and was more potent than monomeric DTEGF, DTIL13, or an equimolar mixture of DTEGF and DTIL13 indicating the importance of having both ligands on the same single chain molecule. Binding studies of FITC labeled DTEGF13 on MiaPaCa-2 cells showed a K_d of 56 nM (Figure 1C). DTEGF13 did not inhibit EGF-IL13- Daudi malignant B cells [22] indicating its effects were selective [3].

EGF13 effectively blocks DTEGF13 activity in vitro

In order to determine whether EGF13 engineered without toxin could block binding and killing of DTEGF13, proliferation assays were performed. Figure 1D shows that when as little as 1 nM EGF13 was added to wells containing 1 nM DTEGF13, 17% blocking was observed. The addition of 10 nM blocked over 70% and 100 nM blocked all activity. In previous studies, both anti-EGF and anti-IL-13 antibodies blocked DTEGF13 activity [7].

Toxicity blocking

Having demonstrated that EGF13 blocked DTEGF13 in vitro even at ratios as low as 1:1, studies were performed to determine if EGF13 could block DTEGF13 toxicity in vivo so that we could increase the MTD. Experiments in which normal mice or nude mice were given increasing IP dosing of DTEGF13 showed that the MTD was 0.5 ug/injection or 20 ug/kg (not shown). Thus, in Figure 2A, a group of nude mice were given MiaPaCa-2 tumor cells OT and then on day 7, 9, and 11 post-tumor injection, 7.5 ug of DTEGF13 (a dose 8-10 fold higher than a lethal dose). Half of the animals died after the first injection and the other half died after the second injection never receiving a third. An identical group of animals given a blocking dose of 200 ug or 8 mg/kg EGF13 (an excess of 400-fold) also delivered IP immediately prior to the IP toxic dose of DTEGF13 resulted in full and significant protection from the lethal dose ($p < 0.0003$). In fact, Figure 2B shows that administration of EGF13 and DTEGF13 delivered in this manner could be repeated three times every-other-day (QOD) without any weight loss. This 3 injection QOD schedule is referred to as our standard "single course".

In order to determine if the effect was selective, groups of normal mice in Figure 2C were treated with a single course of toxicity blocking with EGF13 in a different experiment and the results were the same as Figure 2A. All animals given the EGF13 block survived. In contrast, if EGF4 or 2219 bispecific proteins were substituted for EGF13, the animals died in a manner similar to the no EGF13 untreated controls. Because EGF4 could not block, apparently both receptors must be simultaneously blocked. Thus, the ToxBloc was dependent on the selective blocking effects of EGF13, proving that it could block the excessive toxic effect of DTEGF13 ($p < 0.0001$). The histology inset in Figure 3 shows the normal liver of a mouse receiving a course of ToxBloc, but extensive liver damage in a mouse that received control 2219 plus 7.5 ug DTEGF13. In Figure 2D, groups of mice were given the usual IP injection of EGF13 followed about 5 or 30 minutes later with the 7.5 ug injection DTEGF13. A delay of 30 minutes between the EGF13 and DTEGF13 injection still resulted in protection. Together, these data indicate the ToxBloc is highly selective, both ligands must be blocked.

Efficacy in an aggressive orthotopic pancreatic cancer model

To determine whether toxicity blocking could be used as a new form of drug delivery to induce an anti-pancreatic cancer effect, a model was developed in which human MiaPaCa-2 transfected with a luciferase reporter gene was surgically injected OT into the pancreas of nude mice (photo shown in Figure 3). Figure 3A shows the aggressiveness of the model since forty days after pancreatic tumor injection with 4×10^6 cells, organs were removed and imaged for tumor presence. High tumor activity was detected in the spleen, pancreas, liver, testes, and intestines. Lower levels were measured in the heart, lung, and kidneys. This model was chosen in part because human pancreatic cancer commonly metastasizes to liver. The MiaPaCa-2 line also contains a GFP reporter gene so mice were fluorescence imaged and Figure 3A shows the presence of multiple pancreatic tumors (arrow) in the same animal.

Figure 3B shows Experiment 1 in which animals bearing pancreatic tumors were treated with a single course of toxicity blocking (3 injections QOD of 200ug EGF13, 7.5 ug DTEGF13). No reduction in tumor in these mice occurred on day 12 despite the fact that animals were injected on days 7, 9, and 11. However, 3 of 5 (60%) mice showed marked tumor reductions on day 19 indicating that the toxicity blocking does indeed produce anti-pancreatic tumor effects, but these effects require multiple doses. Figure 3B also shows these effects did not occur in the no treatment controls (M1-M5) and control mice given DTEGF13 without EGF13 (M11, M12) died immediately of severe hepatic toxicity.

Having established in Experiment 1 that toxicity blocking produced an anti-tumor effect, but the effect was only transient, multiple treatments were necessary. In Experiment 2 (Figure 3C) mice were given 5 QOD courses of toxicity blocking. The 3 injection courses are shown on the graph and were begun on days 4, 11, 18, 25, and 32. In all cases, a marked tumor reduction occurred over time as determined by a reduction in total bioluminescent activity. The individual and averaged data from the same experiment are shown in Figure 4. In order to compare toxicity blocking to DTEGF13 therapy by itself, another group of mice were given IP DTEGF13 at the MTD of 0.5 ug using the exact same injection schedule (Figure 3C and 4A). Interestingly, although most mice responded to the MTD treatment, most mice relapsed which contrasted to the ToxBloc-treated group which did not (Figure 3C and 4B). Analysis of averaged data in Figure 4C showed tumor size was significantly diminished on day 32 and 39 in ToxBloc-treated mice as compared to no treatment controls ($p < 0.01$). In contrast, tumor growth in mice given IP DTEGF13 at the MTD (Direct IP injection) did not significantly differ from no treatment controls. A fourth group of mice in this same experiment were given EGF13 without DTEGF13 and results verified that EGF13 alone was not effective (Figure 3C).

In other experiments, the anti-tumor efficacy of toxicity blocking was reproducible. Figure 5 shows the imaging data from 2 long-term survivors that were given half as many MiaPaCa-2 tumor cells. Five courses of ToxBloc and DTEGF13 treatment were necessary to entirely eliminate tumor in these animals. The 5 treatment courses were more spread out in these mice. In order to verify that bioluminescent analysis was truly measuring tumor regression, representative mice were taken for histologic analysis.

Tissues were harvested from survivor M42 on day 92. Sections from liver, pancreas, and kidney are shown. No evidence of tumor or toxicity could be found in any of the tissues including gut, kidney, liver, lung, and spleen. Tumor presence was documented in the pancreas, spleen, and liver of no treatment controls. In an additional experiment (Figure 6), a larger group of mice were given 5 courses of ToxBloc and DTEGF13 resulting in a marked reduction of pancreatic tumor. Also, Figure 6 shows the weight data from these mice. Despite, receiving 5 courses (15 injections) amounting to 150 lethal doses of DTEGF13, the animals showed no significant weight loss indicating the regimen was non-toxic.

Together, these findings indicated that although DTEGF13 was highly effective in inducing anti-tumor activity, it was limited by its toxicity. The use of toxicity blocking permitted the administration of high doses of DTEGF13 in excess of the MTD with marked anti-tumor responses resulting in some long-term tumor-free survivors. Lack of toxicity as determined in weight studies correlated with histology findings. Histology findings also correlated with bioluminescent efficacy findings.

Discussion

Based on the limited success finding entirely tumor specific markers for drug targeting, investigators have targeted shared markers on tumors and normal tissues in hopes of identifying a useful therapeutic window of treatment [23]. A major contribution of this work is identifying a new, highly effective drug delivery method for targeting unique bispecific ligand directed toxins to markers highly overexpressed on pancreatic carcinomas that are also expressed to a lesser degree on normal tissue. The ToxBloc method indicates that genetic engineering can be used to clearly reduce toxicity permitting the administration of otherwise lethal doses of targeted catalytic toxins. Also, the consistent, significant reduction in pancreatic tumor burden using ToxBloc was in sharp contrast to relapses that occurred when DTEGF13 was given by direct injection at the MTD (0.5 ug/injection, 15-fold less than the ToxBloc dose) in the same experiment (Figure 3C) using the identical ToxBloc injection schedule ($p < 0.01$). A sophisticated tumor marking and imaging technique was used to measure the effects of toxicity blocking on tumor development in real time against a highly aggressive metastatic pancreatic orthotopic cancer model. Results were verified by histologic analysis. The second contribution of this work is the use of a unique class of targeted toxins directed by bispecific ligands. In previous studies, DTEGF13 had greater activity than their two monospecific counterparts and the presence of both ligands on the same bispecific molecule is responsible for the superior activity of DTEGF13. In this paper, we show for the first time that DTEGF13 can be effective against systemic cancer.

These studies target IL13R and EGFR. IL13R is less broadly expressed on normal tissue than EGFR. IL13R is widely expressed on pancreatic cancer cells [24], and mediates a variety of different effects on various normal cell types including B cells, monocytes, natural killer cells, endothelial cells, and fibroblasts [25]. Likewise, EGFR is highly overexpressed on pancreatic cancer cells and shares expression with normal epithelial tissue [26,27]. We hypothesized that the correct bolus dose of recombinant EGF13 bioengineered without the toxin might provide a toxicity blockade and interfere with binding of high dose DTEGF13 to normal cells. Theoretically, there might still be enough overexpressed EGFR and IL13R on pancreatic cancer cells for an anti-tumor effect. Although this hypothesis is not yet proven, this study's findings support it, since a toxicity blocking dose of EGF13 was identified that permitted us to give a dose of DTEGF13 that exceeds the MTD by about 15-fold. Importantly, this dose which exceeds a lethal dose by 8-10-fold can be repeated 15 times over the course of 30-40 days. The final result is the steady regression of an aggressive metastatic pancreatic tumor in all animals resulting in the elimination of tumor and long-term tumor free survivors in some animals, rarely noted in the literature. Another advantage of the method is that it utilizes IP delivery and is not device dependent. Delivery devices such as pumps and liposomes often introduce another level of complication and regulation.

There are several alternative explanations for the effect. First, EGF13 by itself might be responsible for tumor regression, but experiments in which high dose EGF13 was administered without DTEGF13, the animals showed no sign of tumor regression. Another explanation could relate to circulating receptor. Perhaps circulating EGFR and IL13R in the serum neutralize DTEGF13 in vivo and interfere with its ability to kill pancreatic tumor cells [28]. In this instance, treatment with high dose EGF13 would bind circulating receptor,

freeing more DTEGF13 to target and kill tumor. Toxicity occurring because circulating receptors create toxic complexes with DTEGF13 that are filtered into liver and kidney, would be reduced by excess EGF13 which would neutralize circulating receptor and contribute to a longer serum half-life for DTEGF13 reducing its toxicity. Further studies will be necessary to determine the mechanism of ToxBloc.

Although this is the first time a bio-engineered protein has been used to simultaneously increase efficacy and diminish toxicity, in vivo IP administration of unlabeled antibody prior to the same radiolabeled antibody was previously used to improve localization of the same radiolabeled antibody to tumor in mice [20]. This phenomenon called predosing increased tumor uptake of ^{111}In labeled antibody in flank tumors of mice injected with Raji Burkitt's B cell lymphoma by 162%. Predosing decreased uptake in spleen, while the blood level was significantly greater. Also, pre-dosing with an irrelevant control antibody did not enhance tumor uptake. Interestingly, because antigen was not shed in this model, it was concluded that shed receptor had no role in these observations.

Investigators previously examined the feasibility of targeting toxins with dual ligands [29-31], but this is the first use of dual cytokine targeted toxins. BLT themselves may represent an important advance in the field since only certain combinations of ligands can enhance BLT activity. The observation does not appear to be limited to cytokines, since dual scFv targeting prominent cancer associated markers can also be used. For solid tumors, anti-EpCam in combination with anti-Her2/neu scFvs shows promise as a BLT [5]. For hematopoietic tumor targeting, scFvs targeting CD22 combined with scFv targeting CD19 shows potential for leukemia/lymphoma therapy and has been IND approved by the FDA [32]. The reason that certain combinations of ligands show superiority over others is not yet clear. Studies indicate that it is not strictly attributed to binding, therefore it may be at the level of cellular internalization or perhaps subcellular compartmentalization. Despite the current lack of understanding of the mechanism of toxicity blocking of BLT, it appears to involve saturable competing receptors and it may very well be that toxicity blocking will be useful for improving efficacy and diminishing toxicity of other BLT as well.

The data clearly indicate that multiple injections will be necessary to induce an anti-pancreatic tumor response and prevent the tumor from spreading since it metastasizes in all untreated animals. Recent advances in the toxin field may make multiple injections more feasible. Onda and Pastan recently identified the presence of 7 major epitopes and 13 subgroups responsible for PE antigen recognition by B cells [33,34]. By introducing a total of 8 mutations in all 7 major B cell groups, a PE-based immunotoxin was created with dramatically reduced immunogenicity, but its anti-tumor activity was minimally affected [35,36]. We are currently applying this approach to DTEGF13 and toxicity blocking to reduce its immunogenicity.

In summary, we developed a unique "outside-the-box" delivery approach based on blocking critical receptors on vital organs to reduce toxicity thereby permitting administration of multiple lethal doses of a novel BLT. A sophisticated tumor marking system revealed that ToxBloc was effective in preventing tumor growth and hepatic metastasis in a manner seldom noted in the literature. ToxBloc combined with the use of a superior BLT such as DTEGF13 may provide a unique alternative therapy for drug refractory pancreatic carcinomas. The fact that DTEGF13 works against lethal pancreatic cancer [3] and other lethal cancers such as prostate [7] and glioblastoma [37] enhances the urgency of drug development.

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Figure 1.

A) Construction of DTEGF13. The gene fragment encoding the single-chain BLT DTEGF13 was created using overlap extension PCR. This construct consisted of: 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). The NcoI/XhoI target gene fragment was cloned in the pET21d bacterial expression vector. B) The in vitro activity of BLT DTEGF13 and monospecific DTEGF and DTIL13 was determined by measuring ³H-thymidine incorporation into MiaPaCa-2 cells following 48 hours incubation with varying concentrations of cytotoxins. Data is mean of triplicate samples ±SD and it is expressed as percentage of ³H-thymidine relative to control cells incubated in media alone (% Control Response). IC₅₀ (inhibitory concentration 50%) is the concentration of BLT that inhibits 50% of the untreated control activity. Control counts for untreated Mia PaCa2 cells = 84,429 ± 5,887 cpm/10,000 cells. C) Increasing concentrations of FITC-labeled DTEGF13 were reacted with MiaPaCa-2 cells in order to determine the K_d. D) The ability of genetically engineered EGF13 devoid of toxin to block the binding and killing of 1 nM DTEGF13 to MiaPaCa-2 cells was tested in thymidine incorporation assays mentioned in (A).

Figure 2.

Toxicity Blocking (ToxBloc) studies in mice. A) Toxicity blocking was undertaken to determine whether the toxicity of DTEGF13 could be reduced in vivo so 200 ug of EGF13 was injected into mice IP followed by IP injection of a lethal dose of 7.5 ug DTEGF13 ($N=10/\text{group}$). Controls were given 7.5 ug DTEGF13 without the EGF13 block. The arrows indicate the days of injection. Data is shown as a Kaplan-Meier survival plot and the two curves are significantly different ($P < 0.0003$). B) The average body weight in grams was graphed versus time in days for the animals shown in (A). C) To test the specificity of the ToxBloc, 200 ug negative control peptide 2219 or EGF4 was administered instead of EGF13, prior to the lethal dose of 7.5ug DTEGF13. The 2219, EGF4, and no blocking control curves all were significantly different than the EGF13 ToxBloc curve ($P < 0.0001$). Survival data are shown. D) The effect of varying the interval between injections of EGF13 and DTEGF13 was measured. The 7.5ug DTEGF13 dose was administered IP 5 and 30 minutes after the IP blocking dose of EGF13. 2219 was given as a blocking control. The two EGF13 curves differed significantly from the control 2219 curve ($P < 0.0001$).

Figure 3.

ToxBloc results in anti-tumor efficacy. (A) To study the metastatic ability of the MiaPaCa-2-luc tumor, data are shown from a representative mouse imaged on day 40 following orthotopic injection with MicaPaCa2-luc on day 0. Two images are shown for each organ. The image on the left is without bioluminescent imaging and on the right, the same organ with bioluminescent imaging. Bioluminescence intensity is shown as a function of photons/sec/sr/cm² and is listed for each image. The vector within MiaPaCa-2-luc also contains a GFP reporter gene enabling the fluorescent imaging also shown in (A). The arrow points to several tumor foci in the pancreas. (B) Bioluminescent imaging from Experiment 1 in which nude mice were given an OT injection of 4×10^6 MiaPaCa-2 tumor cells on day 0.

ToxBloc was limited to a single course of treatment (MWF injection) beginning on day 5. Groups included a ToxBloc group, a no treatment control, and mice treated with DTEGF13 without the ToxBloc (which died immediately). C) Bioluminescent imaging from Experiment 2 in which nude mice were given an orthotopic injection of 4×10^6 MiaPaCa-2 tumor cells on day 0. The ToxBloc was administered in 5 courses (15 injections total). Groups included a ToxBloc group, a no treatment control, and a control group given EGF13 devoid of toxin (7.5 ug/injection). A group of mice given IP injection of the MTD of DTEGF13 (0.5 ug) was also tested. Bioluminescence intensity is shown. D) Histopathology studies were performed to determine the effect of the ToxBloc on the liver. Sections were taken from mice given the EGF13 ToxBloc and mice given the DTEGF13 plus control 2219 peptide and stained with H and E. Magnification is 100x. The OT injection of a mouse pancreas is also shown.

Figure 5.

Imaging of an efficacy experiment in which mice were given fewer MiaPaCa-2 tumor cells OT. Bioluminescent imaging from Experiment 3 in which nude mice were given 2×10^6 MiaPaCa-2-luc tumor cells OT on day 0. ToxBloc was administered in 5 courses (15 injections total). Mice were treated with ToxBloc and DTEGF13 or were untreated. Bioluminescence intensity is shown as a function of photons/sec/sr/cm². ND = Not done. Lower panels - Histopathology studies were performed on long-term survivor M42 on day 92. Tissue sections were taken, frozen, sectioned and stained with H and E.

Figure 6.

Total photon tumor activity of the individual mice from an additional experiment in which larger groups of mice receive ToxBloc. A group of 7 mice were given 4×10^6 MiaPaCa-2-luc tumor cells OT on day 0. Mice were either A) not treated as controls or B) treated with 5 courses of ToxBloc plus DTEGF13 delivered QOD MWF. Data are expressed as total activity graphed over time for each individual animal. The curves in (A) and (B) were significantly different. ($p<0.0003$) C) Average body weight of (A) and (B) were compared without any significant variation as determined by Student T test indicating that the regimen was not toxic.