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Improving the *In Vivo* Efficacy of an Anti-Tac (CD25) Immunotoxin by *Pseudomonas* Exotoxin A Domain II Engineering

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Abstract

Tac (CD25) is expressed on multiple hematological malignancies and is a target for cancer therapies. LMB-2 is an extremely active anti-Tac recombinant immunotoxin composed of an Fv that binds to Tac and a 38kDa fragment of *Pseudomonas* exotoxin A (PE38). While LMB-2 has shown high cytotoxicity towards Tac-expressing cancer cells in clinical trials, its efficacy was hampered by the formation of anti-drug antibodies against the immunogenic bacterial toxin and by dose-limiting off-target toxicity. To reduce toxin immunogenicity and non-specific toxicity, we introduced six point mutations into domain III that were previously shown to reduce T cell immunogenicity and deleted domain II from the toxin, leaving only the 11aa furin cleavage site which is required for cytotoxic activity. While this strategy has been successfully implemented for mesothelin and CD22 targeting immunotoxins, we found that removal of domain II significantly lowered the cytotoxic activity of α Tac immunotoxins. To restore cytotoxic activity in the absence of PE domain II, we implemented a combined rational design and screening approach to isolate highly active domain II deleted toxin variants. The domain II deleted variant with the highest activity contained an engineered disulfide-bridged furin cleavage site designed to mimic its native conformation within domain II. We found that this approach restored five-fold of the cytotoxic activity and dramatically improved the maximum tolerated dose. Both of these improvements led to significantly increased anti-tumor efficacy *in vivo*. We conclude that the next generation anti-Tac immunotoxin is an improved candidate for targeting Tac expressing malignancies.

Keywords

Tac; CD25; recombinant immunotoxin; *Pseudomonas* exotoxin A; PE domain II

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Introduction

The alpha chain of the IL-2 receptor, also known as Tac or CD25, is expressed on activated T and B cells, on regulatory T cells and to a lesser degree on sub-populations of resting memory T cells (1–5). Tac is also expressed on multiple T and B cell malignancies and is therefore a target for cancer therapy (6). LMB-2 is an anti-Tac recombinant immunotoxin (RIT) containing a targeting scFv and a 38-kDa portion of *Pseudomonas* exotoxin A (PE38) that shows extremely high cytotoxic activity and has been evaluated in several clinical trials (6–9). One such clinical trial involved various hematological malignancies including adult T-cell leukemia, B-cell chronic lymphocytic leukemia, anaplastic large-cell lymphomas, B-cell non-Hodgkins lymphoma, Hodgkin's disease and hairy cell leukemia (6). In this trial, a general 3% complete remission and a 20% partial remission rate was observed, with 37% of immunotoxin recipients generating anti-drug antibodies and with vascular leak syndrome (VLS) toxicity limiting the dosage.

Parental PE38 consists of PE domains II and III, with the cytotoxic catalytic activity residing within domain III. In order to partially address the problems of non-specific toxicity and immunogenicity of immunotoxins containing the PE38 fragment, we previously engineered a lysozyme resistant version of PE38, designated PE24 or LR (10–12). We showed that all of domain II can be replaced with a short furin cleavage site (FCS) ("RHRQPRGWEQL"), which is necessary for immunotoxin activation after internalization, and is natively found within domain II. These domain II truncated PE24s showed less proteolytic cleavage by lysosomal enzymes and contain less immunogenic sequences immunotoxins than the parental PE38 immunotoxins, and should theoretically be less immunogenic. In addition, PE24 immunotoxins exhibit less non-specific toxicity, allowing much higher doses of immunotoxin to be given (10–12). However, removal of domain II, which does not affect or enhance the cytotoxic activity of immunotoxins targeting the B cell marker CD22 or the mesothelioma marker mesothelin, causes a 30-fold reduction in the activity of α Tac immunotoxins [see Results and (13)].

In order to further reduce the immunogenicity of the bacterial toxin part of the immunotoxin, we previously determined single amino acid point mutations within PE domains II and III that disrupt B (14–16) and T cell epitopes (17–20). While B cell epitope disrupting mutations are intended to avoid recognition by B cell receptors, the T cell epitope disrupting mutations are intended to reduce activation of T helper cells that are necessary for B cell activation. T cell epitopes within domain II and III were mapped by activating human PBMCs with a peptide array spanning domains II and III of PE38 and measuring T cell activation by IL-2 secretion. Once mapped, point mutations that disrupt each epitope without significantly reducing cytotoxic activity were determined. A combination of PE domain II truncation and T cell epitope disruption in PE domain III has previously been introduced into an anti-mesothelin immunotoxin and been found to reduce the activation of human PBMCs *in vitro* by 90% (19) and the formation of anti-drug antibodies (ADA) in mice by 99% (20).

Here we describe the rational design of a next generation version of LMB-2 that combines high cytotoxic activity with low immunogenicity and low general toxicity by introducing six

T cell epitope disrupting point mutations into toxin domain III combined with a deletion of toxin domain II. We found that removal of domain II significantly lowered the activity of Tac mediated intoxication, but not transferrin mediated intoxication in the same cells. To restore Tac mediated cytotoxic activity, we screened a panel of rationally designed domain II truncation mutants. The most active domain II truncated mutant (α Tac-M18-PE24) showed a 5-fold increase in cytotoxic activity, a 68-fold improvement in the maximum tolerated dose (MTD) and significantly increased anti-tumor efficacy *in vivo*. We conclude that the next generation anti-Tac immunotoxin is an improved candidate for targeting Tac expressing malignancies.

Materials and Methods

Vector construction

All protein-encoding sequences were constructed by ordering double stranded DNA fragments (gBlocks, Integrated DNA Technologies, Coralville, IA) codon optimized for *E. coli* and cloning them into the appropriate vector using Gibson Master Mix (New England Biolabs, Ipswich, MA). The ZZ fusion proteins were cloned into the pET22-NN-ZZ-PE38 (kindly provided by Itai Benhar, Tel Aviv University, Israel) which contains the pelB periplasmic leader peptide for periplasmic secretion followed by sequences encoding for two repeats of the Z protein and a multiple cloning site (21). The sequences encoding for refolded RITs were cloned into our standard protein production lab vector (22).

RIT production

All RITs were expressed and purified from *E. coli* inclusion bodies as previously described (23). Briefly, inclusion bodies from *E. coli* were washed and then solubilized using a guanidinium buffer (6M guanidine-HCl, 100mM Tris-HCl, 2 mM EDTA). The soluble denatured proteins were then diluted 1:100 into refolding buffer (100 mL Tris-HCl, 1 mM EDTA, 0.5M arginine, 0.5M NDSB-201, pH 10) and stirred for 32 hours at 4°C. The proteins solution was then dialyzed against 30 mM Tris-HCl, 0.1M urea for 40 hours. The refolded proteins were further purified using ion-exchange and size exclusion chromatography. Protein purity was assessed by SDS-PAGE on a 4-12% Bis-Tris gel (Novex, Life Technologies, Waltham, MA).

ZZ fusion protein production

The ZZ fusion proteins were induced by growing transformed *E. coli* BL21(DE3) in 50 ml of auto-inducing Magic Media (Thermo-Fisher Scientific, Waltham, MA) overnight at 37°C. After growth, the cells were spun for 10 minutes at 5,000 RPM and frozen over night. The pellets were then lysed by re-suspending the cells in BPER complete reagent (Thermo-Fisher Scientific) supplemented with 1 mM EDTA and gentle shaking for 30 minutes at room temperature. The lysate was then cleared by centrifuging for 20 minutes at 12,000 RPM at 4°C and the soluble fraction taken for further purification. Purification of ZZ fusion proteins was accomplished using IgG coated beads (GE Healthcare Life Sciences, Chicago, IL) according to manufacturer's instructions. Finally, proteins were desalted using PD-10 columns (GE Healthcare Life Sciences) and concentrated using Ultracel-4 10-kD cutoff

centricons (Millipore, Billerica, MA). Protein purity was assessed by SDS-PAGE on a 4-12% Bis-Tris gel (Novex, Life Technologies).

Cell growth inhibition assays

The cytotoxic activity of the RITs was evaluated in a cell growth inhibition assay using the WST-8 cell counting kit (Dojindo Molecular Technologies, Rockville, MD). Cells were incubated with varying concentrations of immunotoxin for 72 hours, after which the WST-8 reagent was added and the plates read at OD 450 nm. Readings were normalized to the PBS only positive control and the IC₅₀ (concentration inhibiting growth by 50%) for each construct was calculated using a variable four-parameter slope, non-linear regression fit with Graphpad Prism vs. 6.01. One-hundred percent cell killing was achieved using 100 µg/ml of Cyclohexamide (Sigma, St. Louis, MO) as a control. Each assay contained four replicates of each concentration and the assays were repeated three to four times. Standard RITs were directly added to the cell culture. ZZ-PE fusion proteins were mixed at a 1:1 molar ratio with αTac human IgG1 (Dacluzimab, kindly provided by Thomas A. Waldmann, National Cancer Institute, Bethesda, MD) (24) for 30 minutes at room temperature and then added to the cell culture. HUT-102, the prototype HTLV-1-positive leukemic cell line, was also obtained from Thomas A. Waldmann. KARPAS-299 cells were obtained from Robert J. Kreitman (National Cancer Institute, Bethesda, MD). Identity of all cell lines was confirmed by short tandem repeat testing within the past 12 months and all cell lines were tested negative for mycoplasma. cells were seeded at a concentration of 1 million cells/T75 flask, split when confluent and used after 3-9 passages.

Non-specific toxicity

All animal experiments were performed in accordance with NIH guidelines and approved by the NCI Animal Care and Use Committee (protocol LMB-014). Non-specific toxicity was evaluated by single IV injections of indicated doses to Swiss mice. A dose was considered toxic if mice appeared morbid, loss >10% body weight or died. Morbid and dead mice were sent for necropsy or pathology analysis two days after treatment. Pathological examination included mice weight, hydration, muscularity and histopathological examination of the heart, lungs, spleen, kidneys, gastrointestinal and reproductive tract.

Mouse xenograft tumor model

Female SCID mice were injected subcutaneously in the flank with 4.0×10^6 KARPAS-299 cells. Tumor size was evaluated every other day using a caliper. When tumors reached a range of 62-152 mm⁴, mice were assembled in groups with similar average tumor sizes and treated with IV injections of LMB-2, αTac-dsFv-M18-PE24(T) or vehicle three times (on days 1, 3 and 6). Body weight and tumor size were evaluated in all mice until tumors reached 550 mm⁴. No animals were excluded from statistical analysis. Tumor growth delay was calculated by interpolation of the time point each tumor reached 200 mm³. Kaplan Meier survival curves were calculated based on the fact that mice were euthanized when tumors reached 550 mm³.

Graphs and statistics

Graphs were plotted and analyzed using Graphpad Prism vs. 6.0101 (GraphPad Software, Inc., La Jolla, CA). For differences in tumor growth a one-way ANOVA with Holm-Sidak multiple comparisons test was used, while a Mantel-Cox test was used for the Kaplan-Meier survival graph.

Results

Efficient intoxication through the Tac pathway requires PE domain II

LMB-2 is an α Tac-scFv-PE38 immunotoxin. For this study, we used the same targeting α Tac Fv in our immunotoxins, but in a dsFv format (α Tac-dsFv-PE38) to improve the stability of the antibody fragment (Fig. 1). We compared the cytotoxic activity of LMB-2 and α Tac-dsFv-PE38 by treating HUT-102 cells with the two RITs and found that the two had identical IC₅₀s (Table 1), indicating that disulfide stabilization of the Fv does not affect overall activity.

To reduce the non-specific toxicity and immunogenicity of domain II, we constructed α Tac-dsFv-PE24 (Fig. 1B). The cytotoxic activity of α Tac-dsFv-PE38 and α Tac-dsFv-PE24 was compared by treating HUT-102 and KARPAS-299 cells with the two variants and evaluation of cell viability 72 hours later (Fig. 2A, 2C and Table 1). We found that on HUT-102 cells, the α Tac-dsFv-PE24 immunotoxin was 34-fold less active than α Tac-dsFv-PE38 (IC₅₀s of 3.4 pM and 0.1 pM, respectively). This pattern was more profound on KARPAS-299 cells, where α Tac-dsFv-PE24 was 45-fold less active than α Tac-dsFv-PE38 (IC₅₀s of 10.3 pM and 0.23 pM, respectively). This indicates that PE domain II is important either for killing through the Tac receptor or in general for killing Tac expressing cells.

To further study the role of domain II in Tac-mediated intoxication, we produced PE38 and PE24 immunotoxins that target the ubiquitous transferrin receptor (TfR). This allowed us to target Tac expressing cells through two different endogenous receptors. Comparison of the cytotoxic activity of α TfR-dsFv-PE38 and α TfR-dsFv-PE24 revealed similar cytotoxic activity of α TfR-dsFv-PE38 and α TfR-dsFv-PE24 (Fig. 2B and 2D) with IC₅₀s of 0.77 pM and 0.32 pM, respectively in HUT-102 cells and 0.64 pM and 0.93 pM in KARPAS-299 cells, respectively. These results suggest that the large difference in activity between α Tac PE38 and PE24 immunotoxins is specific to the Tac intoxication pathway and not a general property of Tac expressing cells.

Rational design and screening of PE domain II mutants

In order to isolate a PE domain II truncation mutant containing the minimal amount of domain II residues while retaining high cytotoxic activity, a panel of domain II truncation mutants were rationally designed based on the structure of the full PE38 (PDB ID: 1IKQ) (the most active mutants are described in Figure 1 while the full panel is described in Supplementary Figure S1). By expressing all of our constructs as ZZ-fusion proteins, we were able to produce them by secretion into the *E. coli* periplasm and bypass the laborious protein refolding process necessary to produce standard RITs (21, 25, 26). The production and screening of the ZZ-PE fusion proteins is detailed in Supplementary Figure S2. Briefly,

a preliminary comparison of the cytotoxic activity of ZZ-PE38 and ZZ-PE24 mixed with an anti-Tac IgG mAb showed similar activities on HUT-102 cells, unlike the large difference previously seen between PE38 and PE24 standard RITs. We concluded that the ZZ fusion system does not recapitulate the internal cellular trafficking of RITs, and thus can only be used to discriminate between active/non-active mutants, which must then be produced as refolded immunotoxins and re-tested. Subsequently, the ZZ fusion domain II mutants were mixed with an anti-Tac mAb and screened for cytotoxic activity on HUT-102 cells. Of the active mutants found in this screen, we decided to focus on mutants M12, M14 and M15-M18 based upon a combination of activity and a minimal amount of retained domain II residues (Fig. 1 and Supplementary Fig. S2). M12 was designed to retain helices 6, 7 and a part of domain 1b. M14 retained helices 1, 2 and 3. M15-M18 were designed with extended Gly-Ser linkers around the FCS. Of these, M16 and M18 also included a constraining disulfide bond around the FCS, intended to mimic the native constrained conformation found in domain II (naturally found as a constrained loop within helices H2 and H3 of domain II).

Domain II mutant M18 containing immunotoxin shows improved cytotoxic activity

The selected six domain II mutants (M12, M14 and M15-M18) (Fig. 1) were produced as refolded RITs according to our standard lab protocol and tested for improved cytotoxic activity compared to α Tac-dsFv-PE24 (Table 1). The most active of the domain II mutants, α Tac-dsFv-M18-PE24 exhibited a 5-fold increase in cytotoxic activity compared to α Tac-dsFv-PE24 (IC_{50} of 0.7 pM and 3.5 pM, respectively).

Disruption of T cell epitopes in PE domain III

We have previously published point mutations that disrupt T cell epitopes found within PE domain III (R427A, F443A, L477H, R494A, R505A and L552E) (20). These mutations were introduced into α Tac-dsFv-M18-PE24, producing α Tac-dsFv-M18-PE24(T). The low immunogenicity α Tac-dsFv-M18-PE24(T) was tested for cytotoxic activity to ascertain the effect of the introduced de-immunizing mutations (Fig. 3). As shown in Figure 3, α Tac-dsFv-M18-PE24(T) exhibited a 1.5-fold lower IC_{50} on HUT-102 cells (IC_{50} of 1.1 pM vs. 0.7 pM) and a 3-fold lower IC_{50} on KARPAS-299 cells (IC_{50} of 5.25 pM vs. 16.1 pM) compared to the parental α Tac-dsFv-M18-PE24.

α Tac-dsFv-M18-PE24(T) exhibits low general toxicity

To evaluate the non-specific toxicity of LMB-2 (α Tac-scFv-PE38) and α Tac-dsFv-M18-PE24(T), Swiss mice were treated with single doses of LMB-2 or α Tac-dsFv-M18-PE24(T) (Table 2). We found that LMB-2 was tolerated with no significant weight loss at a dose of 0.35 mg/kg (n=4), but that 0.4 mg/kg was toxic to 2/4 mice. We also evaluated the toxicity of multiple doses by giving three doses in tumor bearing SCID mice (see below). We found that 4/4 mice died after treatment with 0.3 mg/kg of LMB-2, while three doses of 0.25 mg/kg (n=5) were tolerated with only mild weight loss. It should be noted that the Swiss mice were generally about 20% larger than the SCID mice.

On the other hand, α Tac-dsFv-M18-PE24(T) was tolerated even at a 68-fold higher dose of 24 mg/kg (n=4) (54-fold when corrected for molarity). We did not identify the MTD,

however 4/4 mice treated at a dose of 28 mg/kg died 24 hours after injection, indicating that the MTD is between 24–28 mg/kg. Toxicity of multiple doses (three) in tumor bearing SCID mice indicated that the multiple dose MTD for α Tac-dsFv-M18-PE24(T) was 10 mg/kg (40-fold higher than LMB-2, 32-fold when corrected for molarity).

Histopathological examination of morbid or dead mice recognized hepatotoxicity as the main cause of toxicity in 8/8 mice treated with a single dose at LD50 of either α Tac-dsFv-M18-PE24(T) or α Tac-dsFv-PE38. This toxicity has been previously reported in mice treated with LMB-2 (27). Interestingly, α Tac-dsFv-M18-PE24(T) also induced nonspecific toxicity in the gastrointestinal track in 2/4 mice consisting of damage to the duodenum and jejunum.

α Tac-dsFv-M18-PE24(T) exhibits improved anti-tumor activity

To evaluate the anti-tumor activity of α Tac-dsFv-M18-PE24(T), we implanted KARPAS 299 cells into the flank of SCID mice (Fig. 4). Once each tumor reached 62–152 mm⁴, mice were arranged into groups with similar average tumor sizes and treated with IV injections of LMB-2 (α Tac-scFv-PE38), α Tac-dsFv-M18-PE24(T) or vehicle three times (on days 1, 3 and 6) (Fig. 4). We found that while the tumors treated with vehicle grew rapidly, reaching an average of 200 mm⁴ on day 3.1 (n=7), mice that were treated with three doses of 10 mg/kg of α Tac-dsFv-M18-PE24(T) showed a significant delay in tumor growth compared with the vehicle group ($p<0.0001$ in a one-way ANOVA with Holm-Sidak's multiple comparisons test) and with the LMB-2 group ($p=0.0026$), reaching an average of 200 mm⁴ on day 9.4 (Fig. 4A). Mice treated with three doses of 0.25 mg/kg of LMB-2 showed a milder delay in tumor growth reaching an average of 200 mm⁴ on day 5.5 (n=5) ($p=0.04$ compared to the vehicle group).

To model the effect of tumor growth delay on survival, we employed a Kaplan Meier survival analysis based on a euthanasia criterion when tumor size reached >550 mm⁴ (Fig. 4B). We found that mice treated with α Tac-dsFv-M18-PE24(T) had a significantly longer median survival of 12 days compared to vehicle (7 days) and LMB-2 (9 days) ($p=0.036$ in Mantel-Cox test).

Discussion

Immunogenicity and dose-limiting toxicity severely hamper the efficacy of cancer targeting immunotoxins. PE based immunotoxins, being of bacterial origin, are extremely immunogenic, with ADA being produced in patients with an intact immune system after only one cycle of treatment (9). The production of ADA against immunogenic protein therapeutics can lead to a shorter serum half-life, loss of therapeutic efficacy and can cause serious adverse immunological reactions (28–31). The use of low immunogenicity immunotoxins should allow more treatment cycles to be given safely without the need for an additional immunosuppressive regimen, and increase the overall treatment efficacy. Similarly, addressing dose-limiting toxicity should also increase efficacy by allowing higher dosing. Here we have taken a combined approach to produce a low immunogenicity, low toxicity and yet highly active α Tac immunotoxin. This was achieved by combining T cell

disrupting mutations in toxin domain III, deletion of toxin domain II and engineering of a disulfide constrained FCS to replace domain II and restore cytotoxic activity.

Removal of PE domain II as a general strategy for reducing non-specific toxicity

Immunotoxins can cause either specific or non-specific general toxicities. Specific toxicities occur when the target receptor is expressed not only on the target cells but on additional tissues, usually at much lower levels. Specific toxicities targeting the liver, kidneys and the central nervous system have been described for individual immunotoxins (32–35). Non-specific toxicities, on the other hand, are associated with multiple immunotoxins against different targets. The most common non-specific toxicity for PE based immunotoxins in humans is VLS, caused by an unknown interaction between immunotoxins and endothelial cells that can frequently be dose limiting (35–37). Hepatic and renal non-specific toxicities have also been observed (35–37). Removal of PE domain II has been shown to reduce the non-specific toxicity of several immunotoxins, including those targeting CD22 on B cells (10), mesothelin on multiple solid tumors (12) and Tac on T and B cells (this study) in mice. This indicates that removal of PE domain II addresses some common non-specific toxicity and is a general strategy for lowering the toxicity of PE based immunotoxins. It remains to be seen whether this reduction in toxicity seen in mice will translate to humans, and if so, which human non-specific toxicity will be reduced.

The disulfide constrained M18 linker can increase PE24 immunotoxin serum half-life

Part of the reduction in general toxicity seen with dsFv/scFv-PE24 immunotoxins may be attributed to the lower half-life of PE24 based immunotoxins, as their smaller size (51-kD) allows them to be cleared by renal filtration. However, a Fab containing PE24 immunotoxin targeting CD22 (74-kD) that is too large for renal filtration also exhibited a much higher MTD *in vivo* than its dsFv-PE38 immunotoxin counterpart (63-kD) (22). This shows that the reduced half-life is not the main cause of the lower general toxicity and that increasing the serum half-life of PE24 immunotoxins is a viable strategy for increasing their efficacy.

We have previously shown that PE24 based anti-mesothelin immunotoxins containing the standard linear FCS between the targeting Fv and the toxin moiety are subjected to faster serum clearance than ones containing a disulfide constrained FCS (38). The disulfide constrained FCS sequence that showed a longer serum half-life was identical to the M18 mutant used in this study. It is therefore reasonable to expect that α Tac-dsFv-M18-PE24(T) will also have a longer serum half-life than the parental α Tac-dsFv-PE24 as a result of the addition of a protective disulfide bond around the FCS. Additional approaches to further extend immunotoxin serum half-life, such as PEGylation or using a larger Fab targeting moiety, may need to be incorporated into α Tac-dsFv-M18-PE24(T) in the future.

Using α Tac immunotoxins to target regulatory T cells

LMB-2 has also been tested for specific elimination of T regulatory cells (T_{reg}) (9, 39). In melanoma patients, LMB-2 caused a 50% transient reduction in circulating and a 68% reduction in lesion associated T_{reg} cells, but did not improve patient outcomes. As Tac (CD25) is expressed on activated B and T cells in addition to T_{reg} cells (1–5), treatment with an α Tac immunotoxin removes both the unwanted T_{reg} cells and the crucial activated

effector lymphocytes (9). Therefore, α Tac immunotoxins are probably not suited for classic immunomodulation, but may be used to target T_{reg} cells (along with bystander activated lymphocytes) prior to adoptive T cell transfer or administration of an immune checkpoint inhibitor. In such a scenario, several treatment cycles of an α Tac immunotoxin will be given instead of lymphocyte depleting chemotherapy in order to deplete immunosuppressive T_{reg} cells, after which the necessary effector T cells or the immune checkpoint inhibitor will be infused into the patient and be able to function better without T_{reg} immune suppression. In all of these scenarios, the recipient will probably have a mostly intact immune system and will require multiple treatment cycles. Therefore, only a low immunogenicity immunotoxin could be repeatedly administered in order to effectively reduce T_{reg} cells.

In summary, the described next generation immunotoxin α Tac-dsFv-M18-PE24(T) combines high cytotoxic activity and low immunogenicity *in vitro* with low general toxicity and significantly higher anti-tumor efficacy *in vivo* making it an improved therapeutic candidate for treating Tac expressing malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADA	anti-drug antibodies
FCS	furin cleavage site
MTD	maximum tolerated dose
PE38	Pseudomonas exotoxin A
RIT	recombinant immunotoxin
TfR	transferrin receptor
VLS	vascular leak syndrome

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Figure 1. Structural model of PE38 and illustrations of the different α Tac domain II mutants
(A) α Tac-dsFv-PE38 is composed of an α Tac dsFv targeting moiety followed by the PE38 toxin comprising domains II and III. Note the FCS marked in green within PE domain II. The arrow signifies the cleavage site. **(B)** Illustrations of the rationally designed domain II truncation mutants. PE domain II is divided into seven helices, designated H1-H7, with helices H1, H3, H4, H5 and H6 bundled around a hydrophobic core. The FCS (residues 274-284, "RHRQPRGWEQL") naturally resides in helices H2 and H3 of domain II, and is constrained by a disulfide bond formed between Cys265 and Cys287. Mutants were

designed based upon the crystal structure of the full PE38 (PDB ID: 1IKQ). Helixes are marked H1-H7, the FCS (“RHRQPRGWEQL”) is marked as a green box, disulfide bonds are shown as red lines, while asterisks denote solubilizing point mutations (hydrophobic to polar). The numbering system is based upon the mature PE38.

Figure 2. The cytotoxic activity of α Tac PE24 immunotoxins is significantly and specifically lower than α Tac PE38 immunotoxins

Immunotoxins targeting either Tac or the TfR containing either the PE38 or PE24 toxins were tested for cytotoxicity on the HUT-102 (A and B) or the KARPAS-299 (C and D) cell lines in a cell growth inhibition assay. (A) α Tac-dsFv-PE38 and α Tac-dsFv-PE24 were tested on the HUT-102 cell line. While α Tac-dsFv-PE38 had an average IC_{50} of 0.1 pM, α Tac-dsFv-PE24 exhibited an IC_{50} that was 34-fold lower (3.4 pM). (B) In contrast, targeting PE38 and PE24 immunotoxins against the TfR on the same HUT-102 cells yields a very similar IC_{50} for α TfR-dsFv-PE38 and α TfR-dsFv-PE24 (IC_{50} s of 0.77 pM and 0.32 pM, respectively). This difference was also seen when α Tac-dsFv-PE38, α Tac-dsFv-PE24, α TfR-dsFv-PE38, and α TfR-dsFv-PE24 were tested on KARPAS-299 cells (C and D) (IC_{50} s of 0.23 pM, 10.3 pM, 0.64 pM and 0.93 pM, respectively). Representative graphs are shown. Each assay contained four replicates for each data point and the assays were repeated three times.

Figure 3. Disruption of T cell epitopes in α Tac-dsFv-M18-PE24

T cell epitope disrupting point mutations in PE domain III were introduced into α Tac-dsFv-M18-PE24, creating α Tac-dsFv-M18-PE24(T). α Tac-dsFv-M18-PE24(T) was tested on (A) HUT-102 and (B) KARPAS-299 cells in a cell growth inhibition assay. On HUT-102 cells α Tac-dsFv-M18-PE24(T) exhibited a normalized IC_{50} of 1.1 pM compared to an IC_{50} of 0.7 pM for the parental α Tac-dsFv-M18-PE24, while on KARPAS-299 cells α Tac-dsFv-M18-PE24 and α Tac-dsFv-M18-PE24(T) exhibited an average IC_{50} of 5.25 pM and 16.1 pM, respectively. Representative graphs are shown. Assays were repeated 3-4 times.

Figure 4. α Tac-dsFv-M18-PE24(T) exhibits improved anti-tumor activity

SCID mice were implanted with KARPAS-299 cells and then treated with three doses of vehicle (PBS), LMB-2 (α Tac-scFv-PE38) or α Tac-dsFv-M18-PE24(T). Treatment with α Tac-dsFv-M18-PE24(T) causes a significant delay in tumor growth ($p=0.0026$, one-way ANOVA) and in survival ($p=0.036$, Mantel-Cox test) compared to treatment with LMB-2.

Table 1

Cytotoxic activity of the domain II mutant immunotoxins

Construct	IC ₅₀ (pM)	Fold reduction in activity (compared to PE38)
LMB-2 (αTac-scFv-PE38)	0.1	
αTac-dsFv-PE38	0.1	
αTac-dsFv-PE24	3.5	34
αTac-dsFv-M12-PE24	30.2	297
αTac-dsFv-M14-PE24	5.4	53
αTac-dsFv-M15-PE24	1.5	15
αTac-dsFv-M16-PE24	4.2	42
αTac-dsFv-M17-PE24	3.2	32
αTac-dsFv-M18-PE24	0.7	7

Immunotoxins were tested for cytotoxicity in a cell growth inhibition assay on the HUT-102 strain. Averages of 3-6 assays are shown.

Table 2

Maximum tolerated dose in mice

Construct	Dose (mg/kg)	Result	Weight loss (%)
α Tac-dsFv-M18-PE24(T)	18	4/4 healthy	2
	24	4/4 healthy	1
	28	4/4 dead	–
LMB-2 (α Tac-scFv-PE38)	0.3	4/4 healthy	2
	0.35	4/4 healthy	3
	0.4	2/4 healthy	2
	0.5	4/4 dead	1

Swiss mice (n=4) were given a single dose of immunotoxin and monitored for health to establish the maximum tolerated dose.