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A recombinant immunotoxin engineered for increased stability by adding a disulfide bond has decreased immunogenicity

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Recombinant immunotoxins (RITs) are anti-cancer agents that combine the Fv of an antibody against cancer cells with a protein toxin from bacteria or plants. Since RITs contain a non-human protein, immunogenicity can be an obstacle in their development. In this study, we have explored the hypothesis that increasing stability can reduce the immunogenicity of a RIT using HA22-LR, which is composed of an anti-CD22 Fv fused to domain III of *Pseudomonas* exotoxin A. We introduced a disulfide bond into domain III by identifying and mutating two structurally adjacent residues to cysteines at sites suggested by computer modeling. This RIT, HA22-LR-DB, displays a remarkable increase in thermal stability and an enhanced resistance to trypsin degradation. In addition, HA22-LR-DB retains cytotoxic and anti-tumor activity, while exhibiting significantly lower immunogenicity in mice. This study demonstrates that it is possible to design mutations in a protein molecule that will increase the stability of the protein and thereby reduce its immunogenicity.
Keywords: disulfide bond/immunotoxin/immunogenicity

Introduction

The human immune system is essential to protect the body against disease, but can be detrimental under certain circumstances. The therapeutic use of exogenous proteins to treat human disease is one such situation. Many potentially beneficial proteins are recognized as foreign by the human immune system, eliciting the formation of neutralizing antibodies that limit clinical efficacy. Due to increasing interest in the use of proteins as clinical therapeutics, the issue of immunogenicity has become significant. During the development of a therapeutic protein, it is essential to evaluate the protein's immunogenicity and take steps to reduce or eliminate it if necessary (Presta, 2006).

Recombinant immunotoxins (RITs) are protein therapeutics that combine a fragment of an antibody to a tumor-associated antigen with a protein toxin in order to specifically target and kill cancer cells. Several cancer-specific antigens are being used as targets for cancer therapy with RITs. These include CD22, CD25, mesothelin and the IL-3 receptor (Hall *et al*., 1999; Kreitman *et al*., 2000a; Su *et al*., 2009; Prince *et al*., 2010). Immunotoxins now in clinical trials contain portions of diphtheria toxin, *Pseudomonas* exotoxin A (PE) or ricin (Pastan *et al*., 2006, 2007). In our laboratory, we have developed RITs based on PE in which the variable fragment (Fv) of an antibody is fused to a 38-kDa truncated portion of PE, called PE38 (Fig. 1A). Several of these RITs are in clinical trials (Kreitman *et al*., 2000b, 2001, 2005; Hassan *et al*., 2007). The RIT moxetumomab pasudotox (MP), previously known as HA22 or CAT-8015, is being evaluated in the treatment of various B cell malignancies. It contains an affinity-optimized Fv reactive with CD22 fused to PE38. MP has produced complete remissions in ~40% of patients with hairy cell leukemia and complete or partial remissions in children with acute lymphoblastic leukemia (ALL) (Kreitman *et al*., 2001; Wayne *et al*., 2010).

Native PE is a 613-residue (66-kDa) protein made up of three major domains. Domain Ia is located at the N-terminus of the protein and binds to the low-density lipoprotein receptor-related protein 1, which is present on many cell types. In RITs, domain Ia is deleted and replaced with an Fv targeting cancer cells (Hwang *et al*., 1987). Domain II is proposed to assist in the translocation of PE from the endoplasmic reticulum to the cytosol, but does not seem to be universally required for activity (Weldon *et al*., 2009). Domain III is an enzyme that catalyzes the transfer of ADP-ribose moiety from NAD^+ to elongation factor 2 (EF-2), arresting protein synthesis and ultimately leading to cell death (Pastan *et al*., 2007). HA22-LR (Fig. 1A) is a new RIT that contains deletions removing protease sites in domain II (Weldon *et al*., 2009). HA22-LR is resistant to degradation by lysosomal enzymes, and is also less immunogenic than HA22 (Hansen *et al*., 2010). We have recently reported that we have introduced eight mutations into HA22-LR to make HA22-LR-8M, which does not induce antibodies in mice when injected repeatedly by the intravenous route (Onda *et al*., 2008). Although this approach appears promising, we are still interested in exploring other approaches that could be used to decrease immunogenicity of immunotoxins and other proteins.

In the current study, we have sought to reduce the immunogenicity of HA22-LR further by introducing a disulfide bond into domain III. We predicted that a disulfide bond would increase the thermal stability and protease resistance of HA22-LR, leading to less efficient processing and

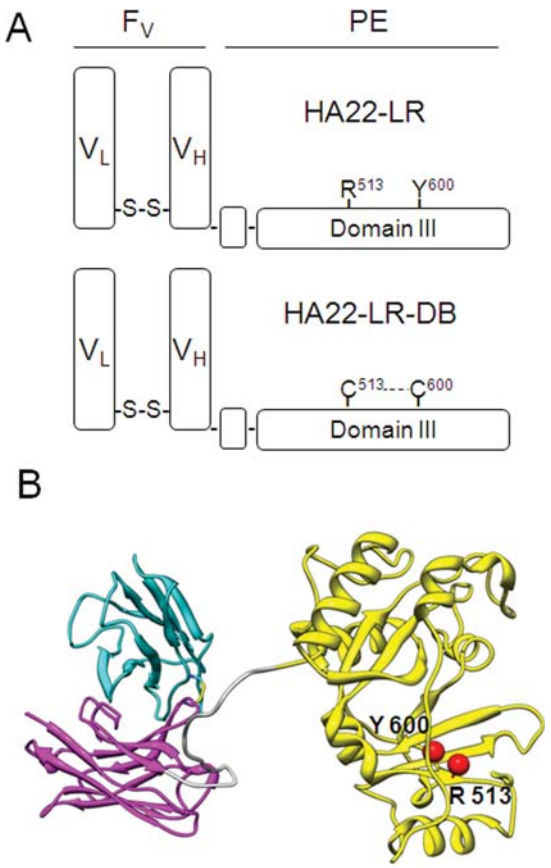

Fig. 1 Construction of HA22-LR-DB. (**A**) Schematic representation of the PE-based RITs HA22-LR and HA22-LR-DB. RITs consist of the Fv of an antibody to a tumor-associated antigen joined with a cytotoxic fragment of PE. The disulfide-stabilized Fv (dsFv) is composed of the V_L covalently linked to V_H through a disulfide bond engineered into the framework region. The V_H fragment is at the N-terminus of a polypeptide chain containing a fragment of PE comprised of the furin protease cleavage site and domain III. The residues chosen for mutation, R^{513} and Y^{600} in HA22-LR, were changed to cysteines in HA22-LR-DB in order to introduce a disulfide bond. (**B**) Structural model of HA22-LR. The V_L is in cyan and V_H is in magenta. Domain III of the toxin is in yellow. The linker containing the furin cleavage sequences is in gray. The location of R^{513} and Y^{600} is indicated. The Fv was modeled using the crystal structure of an antibody fragment (PDB ID 1FBI) and PE was modeled using the crystal structure of native PE (PDB ID 1IKQ). The conformation of the furin-sensitive linker was chosen arbitrarily.

presentation to the immune system. We used molecular modeling to choose a location for the new disulfide bond and produced the new immunotoxin (HA22-LR-DB) in *Escherichia coli*. We show here that HA22-LR-DB retains high cytotoxicity *in vitro* and *in vivo*, is resistant to heat denaturation and proteolysis by trypsin, and is less immunogenic than HA22-LR in mice.

Materials and Methods

Construction, expression and purification

We used computer modeling to choose the positions for a disulfide bond by examining the crystal structure of PE domain III (PDB ID code 1AER) (Li *et al*., 1996). The residues Arg^{513} and Tyr^{600} in domain III were selected and mutated to cysteines using the QuickChange site-directed mutagenesis kit (Stratagene). Mutations were confirmed by sequencing (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems). HA22-LR-DB was expressed in *E.coli* BL21 (λDE3) in a T7-based expression system and purified by the established protocol (Pastan *et al*., 2004). Assays for accessible thiols were performed as previously described to determine the presence of free cysteines in HA22-LR-DB (Ellman and Lysko, 1979; Beer *et al*., 2004).

Activity assays in vitro

CD22-positive human Burkitt's lymphoma cell lines (CA46, Raji, Daudi and Ramos) and ALL cell lines (KOPN-8 and WSU) were used to evaluate the cytotoxicity of RITs. Cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Sigma), 100 U/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen) at 37°C in a 5% CO_2 incubator. The cells were seeded in 96-well plates at 5000 cells in 100 μl per well containing various concentrations of immunotoxin. Viable cell concentrations were determined using Cell Counting Kit-8 (CCK-8; Dojindo, Japan). After 3 days of culture, 10 μl of CCK-8 per well was added and incubated for 3 h at 37°C in a CO_2 incubator. Wells without immunotoxin were used as positive controls for cell viability. A cell-free assay to examine the inactivation of EF-2 was performed as described previously (Pastrana and FitzGerald, 2006) using the TNT quick-coupled transcription/translation system (Promega) and a high-sensitivity β-galactosidase assay kit (Stratagene).

Anti-tumor activity in vivo

A CA46 xenograft tumor model using female severe combined immunodeficiency (SCID) mice was used to evaluate the anti-tumor activity of RITs *in vivo* as described previously (Kreitman *et al*., 1999). Animals were handled according to the National Institutes of Health guidelines approved by the Animal Care and Use Committee of the National Cancer Institute. Tumor sizes were measured by caliper and calculated using the formula: tumor volume (mm^3) = length × $(width)^2 \times 0.4$. When an average tumor size of ~110 mm^3 was reached 6 days after tumor implantation, mice were treated on Days 6, 8 and 10 with intravenous (i.v.) injections of vehicle (200 μl of 0.2% human serum albumin in phosphate-buffered saline) or either of the RITs HA22-LR or HA22-LR-DB (2.0 mg/kg in 200 μl vehicle). Each treatment group consisted of five mice.

Immunization studies

The immunogenicity of HA22-LR-DB was evaluated in Balb/C mice ($n = 10$) as previously described (Onda *et al*., 2008). An immune complex-captured enzyme-linked immunosorbent assay (ICC-ELISA) was performed to measure the titer of antibody (Friguet *et al*., 1985; Onda *et al*., 2006).

Thermal stability

RITs (20 μg/ml) were heated to various temperatures ranging from 45 to 90°C for 15 min using a thermal cycler. Samples were placed on ice immediately after heat treatment and evaluated for activity.

Trypsin digestion

RITs (0.3 mg/ml) were treated with 0.2 μg/ml trypsin (Roche) in 100 mM Tris, 1 mM $CaCl_2$, 2 mM EDTA, pH

8.5. The reaction was allowed to proceed at 37°C for various times between 0 and 8 h before the addition of tris-glycine sodium dodecyl sulfate (SDS) sample buffer immediately followed by incubation at 85°C for 5 min. Samples were analyzed on an 18% acrylamide gel by tris-glycine SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Invitrogen) and Microwave Blue Coomassie blue protein stain (Protiga, Frederick, MD, USA).

Results

Design and preparation of mutant immunotoxin

To determine the best location for a disulfide bond in domain III, we obtained the coordinates of atoms in the crystal structure of domain III from the Protein Databank (PDB ID 1AER) and searched for residue pairs >20 residues apart in the primary sequence in which the alpha carbon (C_α) to C_α distance was 3.8–7.0 Å and the beta carbon (C_β) to C_β distance was 3.4–4.7 Å. Thirty different pairs of residues were located. We then visually examined each of the 30 possible pairs and rejected all but six, typically because they were located in or close to the active site or because their side chains were oriented in a manner unfavorable for disulfide bond formation.

Here we report the properties of a protein in which one of these pairs, Arg^{513} and Tyr^{600}, was used to generate a disulfide bond in the construct HA22-LR (Weldon *et al*., 2009) (Fig. 1A and B). After constructing, expressing and purifying this new mutant, HA22-LR-DB, we analyzed it by SDS–PAGE under reducing and non-reducing conditions (Fig. 2). The protein was over 95% pure. An assay for free thiol groups showed that there was 0.15 mol of free-thiol per mol of HA22-LR, and 0.25 mol of free-thiol per mol of HA22-LR-DB (data not shown). The difference in free thiol concentration is minimal, and we conclude that a new disulfide bond was generally well-formed, formed between residues 513 and 600.

Cytotoxicity activity

We evaluated the cytotoxicity of HA22-LR-DB on several CD22-positive human B cell lines (CA46, Raji, Daudi, Ramos, KOPN-8 and WSU). Cells were incubated with a series of concentrations of HA22-LR or HA22-LR-DB for 3 days, and cell viability was measured by a WST-8 assay. Representative data for CA46 (Fig. 3A) and Raji (Fig. 3B) are shown, and IC_{50} values for all experiments are presented in Table I. Compared with HA22-LR, HA22-LR-DB was 2–4-fold less active on most cell lines tested, but retained cytotoxic activity on all cell lines and was slightly more active on Ramos cells. Notably, the IC_{50} of HA22-LR-DB was well below the 500–1000 ng/ml level reached in patients currently receiving MP (Kreitman *et al*., 2001).

To determine the ability of the mutant immunotoxin to catalyze the ADP-ribosylation of EF-2, we used a cell-free protein synthesis assay in which the EF-2-dependent synthesis of β-galactosidase is measured to assess the activity of PE (Pastrana and FitzGerald, 2006). As shown in Fig. 3C, HA22-LR-DB and HA22-LR had similar abilities to inhibit the synthesis of β-galactosidase (HA22-LR $IC_{50} = 89.4$ ng/ml; HA22-LR-DB $IC_{50} = 78.2$ ng/ml).

Anti-tumor activity in mice

The anti-tumor activities of HA22-LR and HA22-LR-DB were evaluated using a CA46 mouse xenograft tumor model (Kreitman *et al*., 1999). SCID mice with CA46 tumors were treated intravenously with 2 mg/kg of HA22-LR or HA22-LR-DB on Days 6, 8 and 10 post-implantation. The RITs had similar anti-tumor activities, causing complete regressions of all tumors that lasted ~1 week (Fig. 3D).

Immunogenicity studies

To evaluate the relative immunogenicity of HA22-LR-DB and HA22-LR, Balb/c mice were injected intraperitoneally with 10 μg of HA22-LR-DB or HA22-LR every 7 days for four treatment cycles and bled 7 days after each injection. Serum levels of antibody against the RITs were measured using ICC-ELISA (Onda *et al*., 2008), in which the antigen–antibody interaction takes place in solution before being captured on a plate for detection. As shown in Figure 4, the sera from the first and second bleeds of mice treated with either RITs showed no difference from the phosphate-buffered saline control. The antibody titers of both RITs in the third and fourth bleeds were increased, but significantly lower in mice treated with HA22-LR-DB compared with mice treated with HA22-LR ($P < 0.01$ for the third bleed and $P < 0.05$ for the fourth bleed). This result suggests that the addition of a disulfide bond to HA22-LR decreases its immunogenicity.

Thermal stability

Having shown that HA22-LR-DB retained enzymatic and cell-killing activity, we next examined its thermal stability. Samples of HA22-LR-DB and HA22-LR were heated for 15 min at various temperatures and assessed using a cytotoxicity assay on CA46 cells (Fig. 5). HA22-LR lost significant cytotoxic activity when incubated at 55°C and was completely inactivated at 75°C. In marked contrast, HA22-LR-DB showed a much higher thermal stability, being totally resistant to inactivation at 70°C. Only when heated to 80°C did HA22-LR-DB lose activity. These results demonstrate a significant increase in thermal stability by introducing a disulfide bond into HA22-LR-DB.

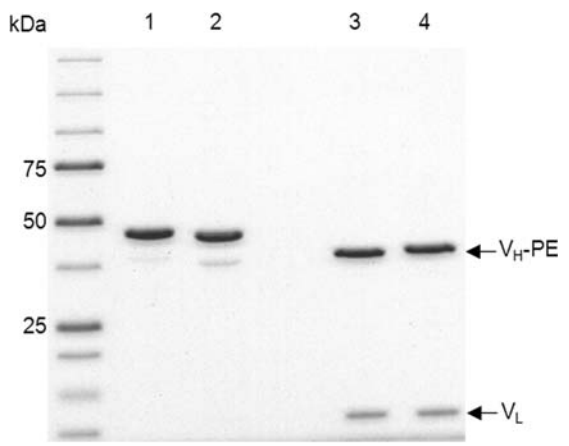

Fig. 2 SDS–PAGE of HA22-LR-DB. Coomassie-stained 4–20% acrylamide SDS–PAGE gel of purified RITs. Lane 1: HA22-LR, non-reducing; lane 2: HA22-LR-DB, non-reducing; lane 3: HA22-LR, reducing; lane 4: HA22-LR-DB, reducing.

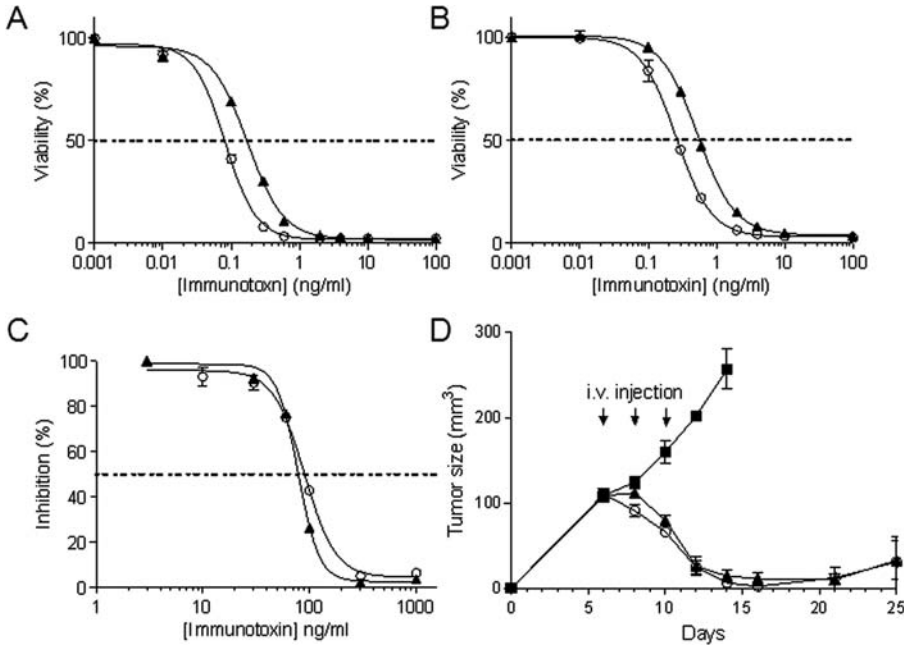

Fig. 3 The activity of HA22-LR (open circle) and HA22-LR-DB (closed triangle) *in vitro* and *in vivo*. The cytotoxicity of HA22-LR and HA22-LR-DB on CA46 (**A**) and Raji (**B**) cells. Cell viability of triplicate cultures was measured after 3 days of incubation, and is plotted relative to an untreated control as the mean value ± the standard deviation. The dotted line indicates 50% viability. (**C**) Cell-free protein synthesis inhibition. Various concentrations of RITs were added to a cell-free eukaryotic β-galactosidase expression system. The activity of triplicate synthesized β-galactosidase was measured, and is plotted relative to an untreated control as the mean value ± the standard deviation. The dotted line indicates 50% inhibition. (**D**) Anti-tumor activity. The anti-tumor activities of HA22-LR and HA22-LR DB were evaluated using a mouse CA46 xenograft tumor model. Mice were treated i.v. with vehicle (closed square) or 2 mg/kg HA22-LR or HA22-LR-DB on Days 6, 8 and 10 (arrows). Tumor size was measured periodically and is plotted here as the mean value of five mice ± the standard deviation.

Table I. Activity of HA22-LR and HA22-LR-DB on CD22-positive cell lines

Cell line	IC_{50} ± SD (ng/ml)		
	HA22-LR	HA22-LR-DB	Relative activity
CA46	0.08 ± 0.004	0.18 ± 0.005	0.47
Daudi	0.13 ± 0.003	0.25 ± 0.056	0.52
Ramos	1.17 ± 0.25	0.73 ± 0.13	1.60
Raji	0.26 ± 0.012	0.54 ± 0.023	0.48
KOPN-8	0.42 ± 0.016	1.24 ± 0.075	0.34
WSU-CLL	0.83 ± 0.032	3.37 ± 0.179	0.25

Stability to trypsin degradation

We evaluated the resistance of HA22-LR-DB to proteolytic digestion using trypsin, a serine protease that preferentially cleaves polypeptide chains after arginine or lysine residues (Olsen *et al*., 2004). The temporal cleavage pattern of trypsin-treated aliquots of HA22-LR and HA22-LR-DB, incubated for up to 8 h, was analyzed by reducing SDS–PAGE (Fig. 6). At the 0 time point both RITs show two bands corresponding to the light chain Fv polypeptide (V_L) (12 kDa) and heavy chain Fv polypeptide (V_H)-PE (39 kDa) portions of the disulfide-bonded fragments of the full-length protein. Fragments of both proteins were generated as the reaction proceeded, but there was a clear delay in the digestion of the 25-kDa domain III fragment of HA22-LR-DB following its anticipated release from the V_H fragment by trypsin cleavage of the protease-sensitive linker (Weldon *et al*., 2009). The data show that the 25-kDa fragment from the HA22-LR-DB was still present after 4 h of digestion in the HA22-LR-DB sample, whereas it was not detected after 2 h treatment of HA22-LR. These results show that the addition of a disulfide bond into domain III of HA22-LR increases its resistance to trypsin degradation.

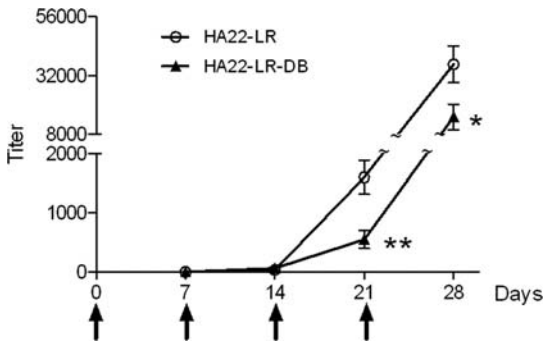

Fig. 4 Immunogenicity of HA22-LR-DB. Serum antibody titers from BALB/c mice ($n = 10$) treated with HA22-LR (open circle) or HA22-LR-DB (closed triangle) were evaluated by ICC-ELISA. Mice were injected intraperitoneally with 10 μg RIT every 7 days (arrows) and bled 7 days after each injection. Antibody titers in serum were measured against their respective RIT using ICC-ELISA. Significance ($*P < 0.05$; $**P < 0.01$) was determined using a Mann–Whitney test.

Discussion

We describe here the production and characterization of a new RI T, HA22-LR-DB. We introduced a disulfide bond into domain III of PE, the domain that catalyzes the ADP-ribosylation of EF-2. The presence of the new disulfide

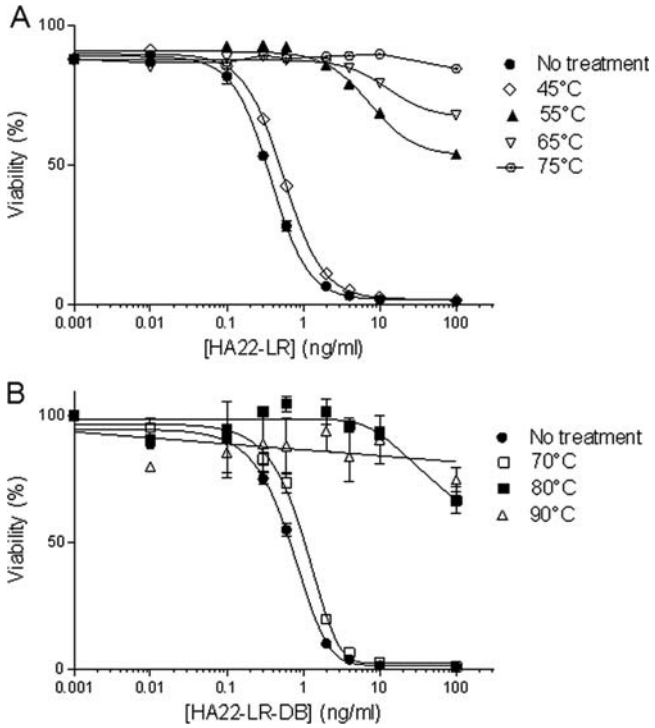

Fig. 5 Temperature stability of HA22-LR-DB. The cytotoxicity of HA22-LR (**A**) and HA22-LR-DB (**B**) on CA46 cells following 15 min incubation at various temperatures was evaluated. Cell viability of triplicate cultures after 3 days was measured and is plotted relative to the untreated control as the mean value ± the standard deviation.

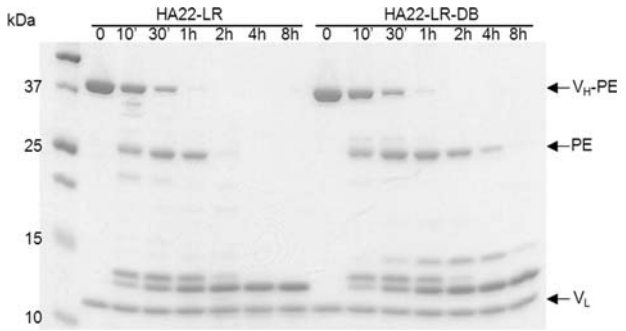

Fig. 6 Time course of RIT degradation by trypsin. HA22-LR or HA22-LR-DB (0.3 mg/ml) is incubated with trypsin (0.2 mg/ml) at 37°C for various times ranging from 0 to 8 h; and analyzed by reducing SDS–PAGE.

bond in the mutant protein was verified using an assay to detect free thiol. The mutant protein retains cytotoxic activity on several CD22+ B cell tumor lines and shows potent anti-tumor activity in mice bearing CA46 xenograft tumors. Its immunogenicity has been decreased and its stability has been increased. We found a large enhancement in thermal stability and increased resistance to trypsin degradation for HA22-LR-DB when compared with its parental molecule, HA22-LR.

Although HA22-LR-DB exhibited the same activity as HA22-LR in a cell-free assay measuring the inhibition of protein synthesis, its activity varied on cell lines. It was generally 2-3-fold less active than HA22-LR on cell lines except for Ramos, where it was more active. We suspect that this difference may be due to the disulfide bond preventing the efficient translocation of PE into the cytosol by decreasing its conformational flexibility. The thermodynamic consequences of the introduction of a disulfide bond have been well studied (Pace *et al*., 1988; Pace, 1990; Wedemeyer *et al*., 2000), but the addition of a disulfide bond does not always lead to protein stabilization. Pace *et al*. analyzed thermodynamic data of ribonuclease T1 to show that one disulfide bond can contribute 3.4 kcal/mol and two disulfide bonds contribute 7.2–9.3 kcal/mol of stabilizing energy (Pace, 1990). Pecher *et al*., however, has reported that only two out of six variants of ribonuclease A with an additional disulfide bond demonstrated enhanced protein stability (Pecher and Arnold, 2009). This result suggests that the position of the disulfide bond is key to increasing protein stability. We used computer-aided rational design to choose the positions for the additional disulfide bond (Reina *et al*., 2002; Mandell and Kortemme, 2009), and determined that Arg^{513} and Tyr^{600} were excellent candidates. The thermal stability of HA22-LR-DB (R513C and Y600C) was remarkably increased over HA22-LR. HA22-LR-DB retained cytotoxicity after heating to 70°C, whereas HA22-LR showed a dramatic loss of activity after heating to 55°C (Fig. 5).

Consistent with increased thermal stability, HA22-LR-DB also demonstrated enhanced resistance to proteolytic degradation. We found that the 25-kDa major degradation fragment of HA22-LR-DB remained visible after a 4 h digestion with trypsin, whereas the same fragment derived from HA22-LR had disappeared by the 2 h digestion point (Fig. 6). We propose that the resistance of HA22-LR-DB to trypsin degradation is due to a conformational stabilization in domain III of PE, caused by the additional disulfide bond, which limits the accessibility of HA22-LR-DB to trypsin. We are planning to use lysosomal extracts from cells *in vitro* or measuring RIT degradation in cells to study the effect on this process. In addition to the enhanced stability, HA22-LR-DB demonstrates significantly lower immunogenicity than HA22-LR in mice, although immunogenicity was not eliminated. The effect of disulfide bond incorporation into the immunotoxin was found to alter the kinetics of antibody formation in mice (Fig. 4), where a pronounced lag was observed compared with its parent molecule HA22-LR. Subsequently, the slopes of the two curves appear more or less parallel. Therefore, the effects of disulfide bond incorporation affect the early stage of antibody formation, and do not necessarily translate into lower steady-state levels once the titers plateau.

In clinical studies, we have observed a correlation between the number of treatment cycles and favorable clinical responses. The results of our clinical trials in hairy cell leukemia showed that most patients need to get 1–4 cycles of the treatment to achieve complete responses (Kreitman *et al*., 2005). Based on these clinical data, the delayed onset of an immune response to HA22-LR-DB could allow patients to receive more cycles.

We believe that the disulfide-enhanced stability may reduce the efficiency of the protein degradation that must precede presentation to T lymphocytes, and subsequent activation of the humoral immune response. However, it is possible that mutations at R513C and Y600C affected the epitope recognition by CD4T cells and consequently reduced the immunogenicity of the RIT. Another possibility is that the disulfide bond introduction changed the conformation of

HA22-LR-DB, which may affect the B cell immune response. Further studies are needed for clarifying these points.

A previous report by Delamarre *et al.* describes a parallel relationship between protease resistance and immunogenicity for ribonuclease-A and horseradish peroxidase (HRP) (Delamarre *et al*., 2006). Protease-resistant forms of ribonuclease and HRP were more immunogenic, which is in apparent conflict with our finding that a more stable protein is less immunogenic. In contrast to our study, Delamarre *et al.* did not attempt to enhance protein stability, but instead created destabilized variants of the two proteins using different mechanisms. Native ribonuclease-A was compared with a less stable variant in which native residues 1–20 were noncovalently bound to a truncated variant missing those residues. HRP was compared with apo-HRP, in which the calcium and heme groups were removed to decrease stability. Here, we used point mutations to generate a disulfide bond that enhanced the stability of a PE-based RIT. Further experiments are needed to understand how the mechanism of stabilization or destabilization contributes to immunogenicity.

In summary, the aim of this study was to decrease the immunogenicity of PE-based RITs used in clinical therapy. We introduced a disulfide bond into the RIT HA22-LR and produced the molecule HA22-LR-DB, which demonstrated enhanced stability to heat and proteolytic degradation. HA22-LR-DB retains good biological activity, and exhibits significantly lower immunogenicity in mice relative to its parent molecule. We believe that a strategy of using disulfide bonds to stabilize proteins can be exploited to reduce the immunogenicity of therapeutic proteins, resulting in increased therapeutic efficacy and potency.

Conflict of interest

The authors declare no conflict of interest. However, I.P., M.O. and B.L. are co-inventors on patents in the development of HA22 for cancer therapy that have been assigned to the NIH.

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