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Recombinant Immunotoxins for the Treatment of Chemoresistant Hematologic Malignancies

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Abstract

Recombinant immunotoxins are proteins composed of fragments of monoclonal antibodies fused to truncated protein toxins. No agents of this class are approved yet for medical use, although a related molecule, denileukin diftitox, composed of interleukin-2 fused to truncated diphtheria toxin, is approved for relapsed/refractory cutaneous T-cell lymphoma. Recombinant immunotoxins which have been tested in patients with chemotherapy-pretreated hematologic malignancies include LMB-2 (anti-CD25), BL22 (CAT-3888, anti-CD22) and HA22 (CAT-8015, anti-CD22), each containing an Fv fragment fused to truncated *Pseudomonas* exotoxin. Major responses were observed with LMB-2 in adult T-cell leukemia, chronic lymphocytic leukemia (CLL), cutaneous T-cell lymphoma, Hodgkin's disease, and hairy cell leukemia (HCL). BL22 resulted in a high complete remission rate in patients with HCL, particularly those without excessive tumor burden. HA22, an improved version of BL22 with higher affinity to CD22, is now undergoing phase I testing in HCL, CLL, non-Hodgkin's lymphoma, and pediatric acute lymphoblastic leukemia.

INTRODUCTION

Evolution of Recombinant Immunotoxins

Toxins are proteins produced by various organisms which kill cells at very low concentrations. Those capable of killing a cell with a single molecule include the bacterial toxins diphtheria toxin (DT) [1], *Pseudomonas* exotoxin (PE), and plant toxins like Ricin [2, 3]. The common pathway for these protein toxins is catalytic inhibition of protein synthesis through prevention of peptide chain elongation within the ribosome. The potent effect of these protein toxins on cancer cells was noted 50 years ago with diphtheria toxin [4]. It was first recognized nearly 40 years ago that diphtheria toxin could be directed selectively to cancer cells by conjugation to antibodies [5]. These were the first generation immunotoxins, composed of protein toxins chemically connected to antibodies. Second generation immunotoxins were made smaller and more specific by chemically connecting the binding fragments of the antibody to fragments of the toxin which are missing the domain required for binding to normal cells [6, 7]. Immunotoxin chemical conjugates were difficult to produce since both ligand and toxin required separate purifications, and the chemical conjugation gave rise to a mixture of molecules varying in the location of the ligand-toxin

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junction, and also in the ratio of ligand to toxin molecules conjugated [8]. To facilitate production for clinical testing, single-chain recombinant immunotoxins were produced from *E. coli* by genetically fusing the necessary toxin fragment of *Pseudomonas* exotoxin with a single-chain antibody [9]. These 3rd generation immunotoxins have a defined ligand-toxin junction and can be produced from bacteria in recombinant form.

Mechanism of Action of Protein Toxins

Plant toxins, including Ricin, Saporin, gelonin, pokeweed antiviral protein, abrin, mistletoe lectin and modeccin, prevent the association of elongation factor-1 and -2 (EF-1 and EF-2) with the 60s ribosomal subunit by removing the base of Adenine⁴³²⁴ in 28s ribosomal RNA [2, 10]. Ricin also removes the neighboring Guanine⁴³²³ [2]. *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT) each ADP-ribosylate EF2 [11, 12]. Either way, peptide elongation within the ribosome is stopped, thus resulting in inhibition of protein synthesis. Plant toxins have rarely been used to make recombinant immunotoxins since the plants producing them generate separate toxin chains for binding and activity. Early attempts to fuse plant toxins to ligands resulted in proteins with impaired ability to kill cells [13]. Bacterial toxins like diphtheria toxin and *Pseudomonas* exotoxin are particularly useful for producing recombinant immunotoxins since such toxins are naturally produced as single-chains containing both binding and enzymatic domains. To make recombinant immunotoxins, the toxin's binding domain is simply replaced by the monoclonal antibody (Mab) fragment.

Intoxication of Cells by PE

The full-length PE molecule is 613 amino acid long and contains three functional domains necessary for cellular intoxication [14, 15]. These include Domain Ia (amino acids 1–252) for binding, domain II (amino acids 253–364) for trans-locating the toxin fragment into the cytosol and domain III (amino acids 400–613) for ADP ribosylating elongation factor 2 (EF-2) in the cytosol. The function of domain Ib (amino acids 365–399) is unknown, but amino acids 365–380 can be removed without loss of cytotoxic activity [16, 17]. A current model of PE-intoxication includes the following steps: 1) The carboxy-terminal residue lysine-613 is removed by a carboxypeptidase in the plasma. 2) Domain Ia binds the $\alpha 2$ macroglobulin receptor on animal cells. 3) The toxin is internalized *via* endosomes into the transreticular Golgi [18]. 4) Once internalized, domain II is proteolytically cleaved between amino acids 279 and 280 by furin [19–21]. 5) The disulfide bond between cysteines 265 and 287, which joins the two proteolytic fragments, is reduced. 6) Amino acids 609–612 (REDL) bind to the KDEL sorting receptor which transports the 37 kDa carboxy terminal fragment from the transreticular Golgi apparatus to the endoplasmic reticulum [22, 23]. 7) Amino acids 280–313 of PE facilitate translocation of the toxin to the cytosol [24, 25]. 8) The ADP ribosylating activity within amino acids 400–602 inactivates the diphthamide residue in EF-2 [11]. The process of ADP ribosylation involves residue His440 and Glu553 [11, 26–28]. His440 binds to NAD *via* hydrogen bonding with O2' of the AMP ribose. The carboxyl group of Glu553 plays the major role in ADP ribosylation through H₂O-mediated hydrogen bonding with Tyr481 and Glu546, allowing Tyr481 to bind NAD through a ring stacking interaction. The target of ADP-ribosylation is a diphthamide residue in EF2, which is a post-translational modification of histidine in EF2 essential for many if not all eukaryotes [29]. The ADP-ribose nucleotide subunit of NAD⁺ is transferred to the NE2

atom of the diphthamide imidazole ring [30]. Inhibition of protein synthesis is sufficient to induce cell death *ex vivo*, but a major component of PE-intoxication is apoptosis resulting from ADP-ribosylation [31–34].

Intoxication of Cells by DT

The full-length DT protein is 535 amino acids long, and is composed of the catalytic A domain (amino acids 1–193), and the cell-binding B domain (amino acids 482–535) [35–37]. In between is the translocation or transmembrane (T) domain [38]. DT is believed to intoxicate cells with the following steps: 1) Proteolytic cleavage occurs prior to cell binding between Arg193 and Ser194 [39], within a disulfide loop formed by Cys186 and Cys201. 2) DT binds *via* amino acids 482–535 to a surface complex of heparin-binding EGF-like growth factor precursor and CD9 [35, 40]. 3) Internalization occurs into an endosome and at low pH the protein unfolds. 4) The TH8 (amino acids 326–347) and TH9 (amino acids 358–376) domains form a hairpin which after inserting into the membrane of the endosome creates a channel for translocation of the catalytic domain to the cytosol [41–46]. Translocation appears to occur from early endosomes [47]. 5) Once in the cytosol, NAD binds to several DT side chains including Glu148, Tyr65, Tyr 54 and His 21, and as in PE-induced intoxication, the ADP ribose of NAD is transferred to EF-2 [29, 48–50]. 6) Also as with PE, apoptosis facilitates cell death [31, 51].

Truncated Forms of Toxins for Making Recombinant Immunotoxins

To fuse PE to single-chain antibodies so that binding to normal cells is minimized, the toxin fragment first used was PE40, which is missing the binding domain amino acids 1–252 [14, 52]. Because it was hypothesized that lowering the number of disulfide bonds would simplify renaturation of recombinant immunotoxins and increase production yield, amino acids 365–380, which contain a disulfide bond within domain 1b, were removed. The resulting 38 kDa truncated toxin PE38 showed no less cytotoxic activity than PE40 [16, 53]. Although production yields were similar whether PE38 or PE40 containing recombinant immunotoxins were used [53], the slightly smaller PE38 fragment became the preferred truncated toxin for PE-containing recombinant immunotoxins. For DT, methionine plus the first 485 amino acids (DAB486) was originally used, since it is missing the binding domain 5 [54]. Removal of a disulfide bond within the T domain improved both cytotoxicity and animal toxicity, and thus the first 388–9 amino acids of DT (DT388, DAB389 or DT390) are now most commonly used [55, 56].

Production of Recombinant Immunotoxins

While *Pseudomonas aeruginosa* and *Clostridium diphtheriae* naturally produce PE and DT respectively, it is more efficient to produce these toxins, particularly their truncated forms, from *E. coli*. For prokaryotic expression, a DNA fragment encoding the recombinant immunotoxin is placed into a plasmid under control of the T7 promoter [57], which allows growth of culture while protein expression is repressed. In late log phase, repression is released by addition of the lactose analog isopropyl-b-D-thiogalactoside (IPTG), allowing production of T7 RNA polymerase, which causes plasmid expression and translation of the recombinant immunotoxin. Although recombinant immunotoxins may be secreted into the periplasm [58] or harvested from the cytoplasm [59] of *E. coli*, we have found that much

greater yields of pure immunotoxins may be achieved by recovery of recombinant protein in insoluble inclusion bodies [60–63]. The advantage of this approach is that insoluble inclusion body protein may be dispersed, washed multiple times with detergent to removed endotoxin, solubilized, denatured and reduced, and then renatured in a redox buffer so that disulfide bonds form properly. After either dialysis or ultrafiltration to lower ionic strength and precipitate aggregates, the pure protein may be obtained by anion exchange and sizing chromatography. This process does not involve affinity chromatography and selectively produces protein which is properly folded. Of the total recombinant inclusion body protein used for renaturation, generally, 5–20% can be obtained pure by this approach. Recombinant immunotoxins have also been produced in a *Pichia pastoris* strain containing mutant EF2 [64, 65].

Recombinant Fusion Toxins

Recombinant fusion toxins, containing single-chain growth factors connected to truncated protein toxins, are often considered recombinant immunotoxins because the ligand, while not an immunoglobulin, is a protein of immunologic interest. The only recombinant fusion toxin approved for clinical use is denileukin diftitox, which is indicated for relapsed/refractory cutaneous T-cell lymphoma (CTCL) and has activity in non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL) [66–68]. Clinical trials have been performed with a variety of fusion toxins containing truncated PE, including CD4-PE40, targeting HIV-infected T-cells [69], TP40, targeting the epidermal growth factor receptor (EGFR) in bladder cancer [70], circularly permuted interleukin-4 toxin targeting glioblastoma multiforme (GBM) and solid tumors [71–74], and interleukin-13 toxin targeting GBM [75–77]. Besides IL2 in denileukin diftitox, a variety of other growth factors have been fused to truncated DT (Fig. 1) and tested in clinical trials, including GM-CSF targeting acute myelogenous leukemia (AML) [78–80], interleukin-3 targeting AML [81–83], and EGF targeting EGFR+ solid tumors [84]. Both DT₃₈₉IL3 and DT₃₈₉GMCSF have produced CRs and PRs in clinical trials, and limiting factors have been cytokine release syndrome, transaminase elevations, and immunogenicity. The remainder of this review will focus on fusions between recombinant antibody fragments and toxins for the treatment of hematologic malignancies.

Types of Fv Fragments Used in Recombinant Immunotoxins

Originally, single-chain antibodies, composed of the variable heavy and light domains fused together *via* a flexible (G₄S)₃ linker, were used to make recombinant immunotoxins [9, 53, 85]. To improve stability, a disulfide bone (Fig. 1) was engineered to link the variable domains by mutating 2 residues which were predicted to be ~5.5 angstroms apart, the size of a disulfide bond. By using a disulfide bone rather than a peptide linker, stability was enhanced for several different recombinant immunotoxins [86–88]. Moreover, the yield of renaturation improved, probably because the disulfide bond prevents transient separation of the variable domains and interactions between variable domains of different molecules. The disulfide-stabilized recombinant immunotoxins exist in 2 chains, one containing VL, and the other containing a fusion of VH with truncated toxin. Still, these immunotoxins are considered recombinant since the disulfide bone forms during redox-renaturation and chemical conjugation is not necessary.

RECOMBINANT IMMUNOTOXINS AGAINST HEMATOLOGIC MALIGNANCIES

Recombinant Immunotoxins Targeting CD25

To target the interleukin-2 receptor (IL2R) present on a variety of hematologic malignancies [89–93], IL2 was originally fused to truncated forms of PE [94–96] and DT [54, 55, 97]. DAB₃₈₉IL2 eventually became approved for use in relapsed and refractory cutaneous T-cell lymphoma and is now known as denileukin diftitox [67]. IL2 binds to the complex of 3 IL2R subunits (CD25, CD122, and CD132) with high affinity ($K_d \sim 10^{-11}$ M) but to CD25 with low affinity ($K_d = 10^{-8}$ M) [98, 99], and CD25 far outnumbers the other subunits, which are often missing, on many types of malignant tumor cells [89, 90]. To target CD25 with high affinity, the anti-CD25 Mab anti-Tac, which binds to CD25 with high affinity ($K_d \sim 10^{-10}$ M), was converted to single chain antibody anti-Tac(Fv) and fused to truncated PE. Anti-Tac(Fv)-PE40 was the first recombinant immunotoxin [9] and the slightly shorter version, anti-Tac(Fv)-PE38, containing the deletion of amino acids 365–380 [53], was developed further under the name of LMB-2.

Preclinical Development of LMB-2 for CD25+Malignancies

Anti-Tac(Fv)-PE38 (LMB-2) and its predecessor molecule anti-Tac(Fv)-PE40 were equally cytotoxic to CD25+ tumor cell lines, activated T-cells, and primary ATL cells [53], and also showed equal antitumor activity toward CD25+ human xenografts in mice [100]. LMB-2 was very cytotoxic toward CD25+ leukemic cells from patients with ATL, hairy cell leukemia (HCL), and T-cell leukemias unrelated to retrovirus [101, 102]. CLL cells were much less sensitive than HCL or ATL cells probably due to lower CD25 expression. Methods have been tested to increase CD25 expression including phosphorothioate oligodeoxynucleotides [103] and cyclosporine [104]. Biodistribution studies showed concentration of LMB-2 into CD25+ tumors *in vivo*, and also showed that the normal organ concentrating LMB-2 most was the kidney [105, 106]. Since mice lack CD25 capable of binding LMB-2, non-human primates were used to predict the toxicity of LMB-2 in man. Cynomolgus monkeys were able to tolerate doses of LMB-2 at 300, 750 and 1000 ug/Kg every other day for 3 days (QOD $\times 3$) with reversible toxicity including loss of appetite and transaminase elevations [101]. Pharmacokinetic experiments in monkeys showed alpha and beta half-lives of 58 and 170 minutes, respectively, similar to the values observed in mice.

Dose-Escalation Study of LMB-2 in Patients with CD25+Malignancies

To determine the activity of LMB-2 in humans, a phase I trial was conducted in 35 patients with chemotherapy-pretreated leukemia, lymphoma and Hodgkin's disease (HD). Dose levels were 2, 6, 10, 20, 30, 40, 50 and 63 ug/Kg QOD $\times 3$. Major responses included 7 partial responses (PR) and 1 complete remission (CR) [107]. The response rate was 40% in those receiving a total cycle dose of > 60 μ g/Kg, vs 0% at lower doses ($p=0.006$). Four (100%) out of 4 patients with HCL responded, with 1 CR and 3 PRs [108]. CR included resolution of all cytopenias and eradication of circulating HCL cells. PR in HCL was associated with 98–99.8% reduction of circulating HCL cells, but not resolution of cytopenias. One patient each with CLL, ATL, CTCL and HD achieved PR [107]. Common toxicities included transaminase elevations associated with fever, suggesting cytokine release [109, 110]. Six out of 35 patients were excluded from further treatment after the first cycle

because of immunogenicity, although 8 CLL patients did not make neutralizing antibodies after a total of 16 cycles. HD patients produced high levels of neutralizing antibodies in 3 out of 11 cases after 1 cycle and in 2 patients after 2–3 cycles of LMB-2. Phase II trials are currently ongoing in CD25+ HCL, CLL and CTCL. Since a significant minority of HCL patients are CD25 negative but all are CD22+, HCL patients requiring additional treatment are being treated first with anti-CD22 recombinant immunotoxin BL22 and HA22 instead of LMB-2.

Introduction to Hairy Cell Leukemia (HCL)

A brief review of HCL is appropriate in view of the activity of LMB-2 and BL22 in this disease. HCL is a B-cell malignancy which presents with pancytopenia and splenomegaly [111–114]. The leukemic cells in the bone marrow and spleen have cytoplasmic projections which look like hairs, and the cells are strongly positive for CD22, CD20, CD11c, and also express CD103 without CD23 or CD5 [115, 116]. The median survival without treatment is 4 years [117], and early treatments like chemotherapy, splenectomy and interferon have minimal if any effect on survival [118, 119]. HCL is remarkably sensitive to purine analogs pentostatin and cladribine, with CR rates of 70–87% and 7–8 year disease free survivals (DFS) of 60–76% [120–128]. However, these treatments are not curative as evidenced by lack of plateau on the DFS curve [120, 121] and highly sensitive PCR assays [129–131]. About 10% of patients have a poor-prognosis variant (HCLv) which is negative for CD25, HC-2 and CD123, and positive for CD27 [132–137]. CRs of HCLv to cladribine have not been reported, and PR rates are only 50% [134, 136, 138–140].

Preclinical Development of Anti-CD22 Recombinant Immunotoxin BL22

CD22 was previously targeted using 2nd generation immunotoxin chemical conjugates containing ricin A-chain, which induced responses and were associated with dose-limiting vascular leak syndrome (VLS) [141–144]. To target CD22 with *Pseudomonas* exotoxin, chemical conjugates were made with truncated PE and either of 2 anti-CD22 Mabs, LL2 and RFB4 [145–147]. Unlike LL2, RFB4 was stable enough as a single-chain Fv to make an active recombinant immunotoxin [147]. For improved stability, the recombinant immunotoxin RFB4(Fv)-PE38 was converted to the disulfide stabilized immunotoxin RFB4(dsFv)-PE38, by mutation of Arg44 of V_H and Gly100 of V_L to cysteines, and removal of the disulfide bond [148]. The resulting recombinant immunotoxin BL22 induced complete remissions in mice with human CD22+ B-cell lymphoma xenografts. The plasma levels achieved in the cured mice could be tolerated in *Cynomolgus* monkeys [149], who, unlike mice, had CD22 on normal B-cells capable of binding RFB4 and RFB4(Fv). Freshly obtained leukemic cells from patients with CLL and NHL were sensitive to BL22 [150], indicating that cells with far fewer CD22 sites/cell compared to cell lines were still capable of being killed.

Phase I Trial of BL22 in B-Cell Malignancies

BL22 was administered to 46 patients with HCL, B-cell lymphomas and leukemias at dose levels of 3, 6, 10, 20, 30, 40 and 50 ug/Kg QOD $\times$ 3 [151, 152]. A total of 265 cycles of BL22 were administered to the 46 patients on study, with up to 33 cycles/patient. Of these 46 patients, a total of 31 had HCL, pretreated with 1–6 (median 3) separate courses of purine

analog, including 1–3 courses (median 2) courses of cladribine. Patients had to require treatment based on either neutrophils $< 1000/\text{mm}^3$, hemoglobin $< 10 \text{ g/dL}$, platelets $< 100,000/\text{mm}^3$, circulating HCL cells $> 20,000/\text{mm}^3$, or tumor-related symptoms. BL22 was markedly effective with 19 (62%) CRs and 6 (19%) PRs, for an overall response rate (ORR) of 81%. Although not required by protocol, all major responders had improvements in cytopenias. Of the 19 CRs, most (53%) had never experienced CR with cladribine, and another 26% had no response to the last course of cladribine. All 3 patients with the poor prognosis variant HCLv [136] had CR to BL22. CR was achieved in 11 after cycle 1, and in 8 after cycles 2–9. CRs required absence of HCL in the bone marrow biopsy by H/E staining, but patients with small clumps of CD20+ cells in the marrow could be classified as CR with minimal residual disease (MRD). Compared to CR without MRD, CR with MRD in the bone marrow biopsy by immunohistochemistry has a higher risk of early relapse [153]. Only one patient in CR with BL22 did not clear MRD in the bone marrow biopsy [152]. At a median follow-up time of 36 (range 5 to 67) months, 7 patients were still in CR. Immunogenicity was reported in 11 (24%) out of the 46 patients. The pharmacokinetics of BL22 were strongly affected by tumor burden since CD22 receptor expression (median 44,000 sites/HCL cell) is high compared to the number of molecules administered. Therefore, plasma levels usually increased on subsequent cycles after patients responded. In HCL, DLT consisted of a cytokine release syndrome in one patient who had fever, bone pain, hypotension, and weight gain (VLS) but no pulmonary edema, resolving in 3 days. The most common serious toxicity in HCL was a completely reversible hemolytic uremic syndrome (HUS), observed in 4 patients, confirmed by renal biopsy. HUS presented with hematuria and hemoglobinuria by day 8 of cycle 2 or 3. These patients received 6–10 days of plasmapheresis but not dialysis and had complete resolution of renal function and correction of thrombocytopenia and anemia. Of these 4 HUS patients with HUS, 3 achieved CR and all resolved both HCL and HUS related cytopenias.

Conclusions of Phase I Testing and Preparation for Phase II Testing of BL22

It was concluded that BL22 is the first agent since purine analogs reported to cause CR in a high percentage of patients with HCL. Its ability to induce CR in chemoresistant patients is due to preservation of strong CD22 positivity despite purine analog resistance. The maximum tolerated dose (MTD) of BL22 was 40 ug/Kg QOD $\times 3$ for cycle 1, which exhibited no unexpected safety concerns in any of the 11 HCL patients begun at this dose level. HUS occurred only during retreatment but was observed at dose levels of 30–50 ug/Kg QOD $\times 3$. It was noted that HUS was absent in HCL either at dose levels under 30 ug/Kg QOD $\times 3$ or on cycle 1. Moreover, 3 of the 4 HCL patients with HUS to BL22 had prior splenectomy, suggesting that these patients had less CD22 sink. BL22 plasma levels and AUCs were inversely proportional to tumor burden. It was therefore decided to begin the phase II trial at 40 ug/Kg QOD $\times 3$, and for subsequent cycles, administer only 30 ug/Kg QOD $\times 3$. This type of dosing was used in 2 HCL patients treated by special exemption before phase II testing began, and of these 2 patients, 1 had a PR after 2 cycles, complicated by HUS on the 2nd cycle [152]. It was also noted that during phase I testing the BL22 cycle causing HUS began relatively soon after the prior cycle, specifically 21–23 days after the last dose in 2 patients, and 16 days after the last dose in 3 others. It was also noted that ADAMTS-13 activity [154] was not significantly impaired in patients diagnosed with HUS

from BL22, suggesting the absence of ultralarge multimers of von Wille-brand's factor which would need to be removed. To avoid HUS during phase II testing, we decided to use only 1 cycle and retreat only if necessary to achieve the normal blood counts necessary for CR. Patients achieving this hematologic remission (HR) would need to have neutrophils $1500/\text{mm}^3$, Hgb 11 dL, and platelets $100,000/\text{mm}^3$. We hypothesized that delaying retreatment would allow more time for healing of subclinical damage to the glomerular endothelium, and thus decrease the risk of HUS. We therefore decided to wait 8 weeks after starting cycle 1 to assess response. Patients in HR would undergo bone marrow biopsy to determine if they were in CR, but regardless would be observed without retreatment. In contrast, patients not achieving HR by 8 weeks would begin cycles of BL22 at 30 ug/Kg QOD $\times 3$ every 4 weeks, until 2 cycles past CR.

Phase II Testing of BL22 in HCL

Thus, the phase II trial was designed with 2 goals in mind. The first was to evaluate the response rate of 1 cycle of BL22, given at the MTD of the phase I trial, 40 ug/Kg QOD $\times 3$. The 2nd goal was to determine whether selectively retreating only those patients without acceptable response to 1 cycle could minimize toxicity and allow patients to achieve durable CR. Eligibility was similar to that in the Phase I trial except that patients had to have < 2 year response after their 1st course or < 4 year response to their 2nd or later course of cladribine. It is generally considered that patients with this level of resistance to cladribine should not be retreated with purine analog and should instead try alternative biologic therapy. Patients received BL22 at 40 ug/Kg QOD $\times 3$, and those not achieving HR 8 weeks later were retreated at 30 ug/Kg QOD $\times 3$ at 4-week intervals until 2 cycles past a CR. To be considered a CR, patients had to maintain normal blood counts consistent with HR for at least 4 weeks and after this time have a negative bone marrow biopsy. To be considered a PR, patients had to have 50% improvement in tumor burden and 50% improvement in normal blood counts.

Results of Phase II Testing of BL22 in HCL

One cycle of BL22 produced 9 (25%) CRs, 3 HRs, and 6 PRs, for an ORR of 50% [155]. Of the 36 patients, 56% were retreated, and the final response after a total of 131 cycles in the 36 patients was 17 (47%) CRs, 5 (14%) HRs and 4 (11%) PRs for an ORR of 72% [155]. Of the 17 CRs, MRD in the bone marrow biopsy was detected by immunohistochemistry in 3 (18%) patients. No patients receiving multiple cycles of BL22 had MRD with CR. At a median follow-up of 26 months, 4 (24%) of the 17 CRs relapsed, including only 1 of the 3 CRs who did not clear MRD from the bone marrow. All 5 patients who received at least 2 consolidation cycles past disappearance of MRD remained in CR, versus none of 3 patients receiving less consolidation cycles ($p=0.018$). Therefore patients who need more than 1 cycle for CR might be in a higher risk group and need consolidation cycles for CR durability. Response was related to spleen status in that patients with spleens < 200 mm had higher CR rate after 1 cycle (41% vs 0%, $p=0.006$) or 1 cycle (64% vs 21%, $p=0.019$) compared to patients with either larger spleens (> 200 mm) or prior splenectomy. Similarly, in patients with spleens < 200 mm, ORR was higher after 1 cycle (73% vs 14%) or 1 cycle (95% vs 36%) than in patients with either spleens > 200 mm or prior splenectomy. It is possible that HCL is more susceptible to BL22 penetration in moderately enlarged than in

massively enlarged spleens. Moreover, in patients with prior splenectomy, disease may be further advanced and HCL cells may be more tightly packed in the bone marrow or lymph nodes. Therefore BL22 experimental therapy should be undertaken optimally before the onset of massive splenomegaly or splenectomy. Nevertheless, CR with BL22 is clearly possible despite massive splenomegaly or splenectomy.

Toxicity, Immunogenicity and Pharmacokinetics of BL22 During Phase II Testing

As in phase I testing, evidence of mild vascular leak was common whether patients were receiving cycle 1 at 40 ug/Kg $\times$ 3 or retreatment at 30 ug/Kg $\times$ 3, with grade I-II hypoalbuminemia (70–81%), edema (42–55%), myalgias (39–45%), nausea (22–40%), and hypotension (14–20%). However, significant pulmonary edema did not occur, probably because of the short plasma lifetime of BL22. In fact, patients could easily tolerate prophylactic fluid before and after each dose, and during the week of infusion, to maintain renal blood flow despite mild 3rd spacing. Also common were transaminase elevations in 60–70% of patients, although they were mostly low grade. Even the rare grade 3 transaminase elevations were not considered dose-limiting per protocol, due to the lack of hepatic dysfunction. On study, only 2 (6%) patients had dose-limiting HUS, although a third patient had grade 1 HUS with normal creatinine. As in Phase I, all HUS cases in HCL patients completely resolved. Immunogenicity was observed in 4 (11%) patients and some received up to 10–13 cycles without immunogenicity. Plasma levels after BL22 infusion decreased with median half-lives of 131–157 min during cycles 1–3, and area under the curve (AUC) was inversely proportional to tumor burden, either by log of HCL count in the blood ($p = 0.0001$ – 0.045), or spleen size ($p = <0.0001$ – 0.007), whether patients were receiving initial or repeated cycles. An example of the gradual increase in AUC with multiple cycles of treatment in a patient with massively enlarged spleen is shown in Fig. (2). Initially, plasma levels are barely detectable, then appear to decrease biexponentially, and finally when disease is minimal or eradicated, monoexponential decreases in levels are observed.

Conclusions of BL22 Testing in HCL

The high activity of BL22 against chemotherapypretreated and often resistant HCL observed during phase I testing was confirmed during phase II. As shown in Table 2, despite using fixed dosing for phase II, the average dose/cycle was similar for phase I and II at 29–33 ug/Kg $\times$ 3. The major difference with selective retreatment employed during phase II was in the lower number of cycles per patient, averaging 3.6 with Phase II vs 8.6 for phase I ($p < 0.0001$). This was expected to lead to lower response rates, but the differences in CR (47% vs 61%) and ORR (72% vs 81%) were not statistically different. Compared to phase I, the dose-limiting HUS rate on phase II was less than half and the immunogenicity less than one-third ($p = 0.021$). The HUS rates were not statistically different due to the low number of HUS cases in both trials. Nevertheless, selectively retreating those patients without HR to 1 cycle of BL22 may minimize toxicity without significantly compromising efficacy.

Targeting CLL with Anti-CD22 Recombinant Immunotoxins

As reported in the phase I trial [152], BL22 is less active in CLL, with only marginal responses observed unrelated to steroids. The lower response in CLL is attributed to the

greatly reduced CD22 expression, with a median of 1250 sites/cell on CLL vs 44,000 sites/cell on HCL cells. Although one or several molecules of toxin are sufficient for cell death, several hundred may need to bind to the CLL cell in order to get one molecule into the cytosol, due to the inefficiency of the multiple steps of cellular intoxication. An important problem is that because the off-rate of BL22 on CD22 is significant, only a fraction of those molecules binding actually internalize. To address this issue, the binding affinity of BL22 was increased by lowering its off-rate. Somatic mutational ‘hot-spots’ in the CDR3 domain of the heavy chain were mutated and screening by phage display resulted in a molecule with 15-fold improved binding. [156]. By mutating residues 100, 100a and 100b of VH from SSY to THW, the cytotoxicity toward CLL cells improved a median of 8-fold. The high affinity variant of BL22, called HA22 or CAT-8015, is now undergoing multicenter testing in separate phase I trials in CLL, HCL and NHL. So far, CAT-8015 is highly active in HCL and out of 23 patients, has resulted in only 1 case of HUS which was too mild to be considered dose-limiting. Meanwhile, preclinical development continues to improve HA22 with respect to binding affinity, immunogenicity, and animal toxicity [157–160].

Targeting Both CD22 and CD19 with a Recombinant Immunotoxin

Dr. Vallera and colleagues have recently reported a bispecific single-chain immunotoxin containing methionine plus the first 389 amino acids of DT, followed by Fv fragments of anti-CD22 and anti-CD19 Mabs [161]. The resulting recombinant immunotoxin DT2219 (Fig. 1) was cytotoxic to CD22+/CD19+ Daudi cells with an IC₅₀ of 2 nM, or approximately 200 ng/ml. The same SSY-> THW mutation in HA22 was made to the anti-CD22 Fv of DT2219, and the average IC₅₀ was decreased to 0.3 nM or approximately 30 ng/ml. DT2219 showed antitumor activity in a Daudi xenograft model with significant decrease in the growth of tumors in nude mice [161]. Moreover, a SCID mouse model showed significant ($p < 0.008$) improvement in survival with DT2219 [161]. DT2219 is now entering clinical testing in patients with CD22+/CD19+ hematologic malignancies.

Targeting CD3 with Recombinant Immunotoxin

The anti-CD3 Mab UCHT1 was first described nearly 30 years ago [162]. UCHT1 was chemically conjugated to a toxin and the immunotoxin was shown to deplete human CD3+ T-cells *ex vivo* and thus prevent graft versus host disease [163]. To target DT to CD3+ cells, the recombinant immunotoxin DT390-UCHT1-sFv was constructed [164]. Although cytotoxic, there was a 22-fold loss of binding affinity compared to the sFv alone. To improve binding affinity, the divalent recombinant immunotoxin A-dmDT390-bisFv was constructed, containing an N-terminal alanine, DT amino acids 1–389, the connector ASAGGS, and UCHT1 VL-VH-VL-VH with 3 (G₄S)₃ linkers between the 4 variable domains [165]. The toxin used in this construct is a double-mutant containing S18A and N235A mutations to prevent glycosylation during production in CHO cells [165]. The plasmid was expressed in a *Pichia pastoris* strain containing mutant EF2 [64], and the protein harvested by 0.1 μ m hollow-fiber micro-filtration. A-dmDT390-bisFv(UCHT1) was purified by Butyl 650 M hydrophobic chromatography, followed by Borate Poros 50 HQ chromatography to remove glycoproteins, and finally by Poros 50 HQ anion exchange chromatography [65]. A total of 11.36 g of pure protein in 8.54 L was produced from 16.32 g of culture supernatant [65]. A-dmDT390-bisFv(UCHT1) at 56.25 μ g/Kg twice daily for 4 days in squirrel monkeys had a

plasma lifetime of 18 min and produced transient transaminase elevations, lethargy, and weight loss, but no decrease in hepatic, renal or hematologic function [166]. A-dmDT390-bisFv(UCHT1) is currently undergoing phase I/II testing in patients with T-cell malignancies.

LIMITATIONS AND CHALLENGES

Immunogenicity Due to Immunogenic Epitopes

One of the major challenges of recombinant immunotoxins is their immunogenicity, since the toxins are derived from bacteria. Although this is much more of a problem for toxins targeting solid tumors, because these patients are more immunocompetent, it remains a major problem for hematologic tumors [107, 152, 155]. One way to address this problem is to determine the immunodominant epitopes on the toxin and alter them so that they become non-immunogenic without altering toxin function [167–170]. A recombinant immunotoxin derivative of HA22 with 8 mutations, 2 in domain II and 6 in domain III, has been found to be fully cytotoxic and less immunogenic to mice [170]. Another way to remove immunogenic epitopes is simply to remove large sections of the toxin which are determined not to be required for activity. A new derivative of HA22, termed HA22LR, contains only residues 274–284 and 394–613 of PE, is resistant to lysosomal degradation, is much more cytotoxic to CLL cells than HA22, and is >10-fold less toxic than HA22 to animals [160].

Immunogenicity Related to the Patient's Immune System

A second general approach is to nonspecifically immuno-suppress the patient so that antibodies are not produced. There was no immunogenicity toward BL22 in patients with CLL, compared to > 1/3 of patients with HCL [152, 155]. Similarly, LMB-2 in phase I testing did not produce neutralizing antibodies in CLL patients, but did in most of the patients with T-cell malignancies [107]. Thus if immune function can be lowered just to the level of a CLL patient, recombinant immunotoxins like LMB-2 and BL22 may not be immunogenic. To test this hypothesis, a clinical trial has been initiated at NIH in which ATL patients are treated with LMB-2 after fludarabine and cyclophosphamide to prevent immunogenicity.

Non-Immunogenic Toxins

To avoid immunogenicity altogether, other approaches may include targeting with non-immunogenic 'toxins', like ribonuclease, Granzyme, monomethyl auristatin E and calicheamicin [171–180]. However, it is not clear that any of these molecules are able to kill cells with one molecule in the cytoplasm. Although preclinical data may be promising when using cell lines expressing 10^4 – 10^6 sites/cell, clinical data may be much less positive because when receptor expression is lower, an insufficient number of molecules are internalized to kill the cell. An additional problem with natural products like calicheamicin is that multidrug-resistant cells may be resistant to the toxin used [181, 182]. It is not clear that PE and DT can be replaced with alternative recombinant toxins and still kill target cells with limited numbers of receptors. If not, their immunogenicity can hopefully be avoided using the approaches outlined above.

Toxicity to Normal Tissues

While the smaller recombinant immunotoxins avoid the nonspecific severe VLS observed with the 2nd generation IgG-toxin chemical conjugates, several have shown potentially limiting directed toxicity to normal tissues. An important principle is that while naked Mabs may be safe in targeting antigens also expressed by normal tissues, recombinant immunotoxins are much more likely to kill these cells and this could limit efficacy. Examples include Erb-B2 targeting liver [183] and GM-CSF targeting normal monocytes and macrophages [80]. The HUS from BL22 is also thought to be antigen (CD22) mediated because this toxicity is not seen with other PE38-containing recombinant immunotoxins [152, 155, 184]. Directed toxicity in some cases is best dealt with by choosing a different antigen to target. However, in some cases where the recombinant immunotoxin is lifesaving, like with BL22 in HCL, it makes sense to determine how to avoid the directed toxicity. The dual strategy of designing new recombinant immunotoxins to target new or old antigens more effectively, and of understanding mechanisms of toxicity to prevent side effects, should facilitate the clinical development of recombinant immunotoxins for the treatment for hematologic malignancies in the future.

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Fig. (1). Change in BL22 pharmacokinetics with HCL tumor burden.

A patient with spleen measuring 250 mm in caudocranial diameter was treated on the phase II trial and plasma levels were measured on day 1 (upper left) and 5 (upper right) of each of 7 cycles. The patient achieved CR with normal spleen size prior to the 7th cycle. Peak plasma levels and AUCs increased with decreasing tumor burden, while the volume of distribution (Vd) decreased.

Fig. (2). Recombinant immunotoxins tested in patients with hematologic malignancies.

Fusion toxins on left contain DT amino acids 1–388 or 389, which are composed of the activity (A) chain, the transmembrane (T) domain, and part of the binding domain (B). Ligands include interleukin 2 (IL-2), 3 (IL-3), or granulocyte-macrophage colony stimulating factor (GM-CSF). DT-containing recombinant immunotoxins include DT1922, containing sFvs against CD22 and CD19, and A-dmDT390-bisFv(UCHT1), containing 2 anti-CD3 sFvs in tandem. Recombinant immunotoxins also contain PE amino acids 253–364 and 381–613 preceded by Fvs against CD25 (LMB-2) or CD22 (BL22 or HA22). In BL22 and HA22, V_L and V_H are disulfide bonded together using engineered cysteine residues replacing Arg44 of V_H and Gly100 of V_L. The linkers between variable domains are composed of (G₄S)₃. HA22 is a high affinity variant of BL22 which contains THW replacing SSY at positions 100, 100a and 100b of V_H. The same point mutations are present in the RFB4(V_H) domain of DT2219. S18A and N235A are point mutations in A-dmDT390-bisFv(UCHT1) which prevent glycosylation during its Eukaryotic expression.

Table 1.

Trials of Recombinant Immunotoxins in Hematologic Malignancies

Recombinant Immunotoxin	Antigen	Toxin	Evaluable Patients Tested	Responses	References
Denileukin diftitox	IL2R	DT	71 CTCL (pivotal)	10% CR, 20% PR	[185]
Denileukin diftitox			45 NHL	7%CR, 18% PR	[186]
Denileukin diftitox			22 CLL	4% CR, 23% PR	[68, 187]
DT388GMCSF	GMCSFR	DT	31 AML	3% CR, 6% PR	[80]
DT389IL3	IL3	DT	40 AML, 5 MDS	2% CR, 4% PR	[83]
LMB-2	CD25	PE	11 HD, 6 NHL, 4 HCL, 3 PTCL, 1 CTCL, 2 ATL	3% CR, 20% PR	[107]
BL22	CD22	PE	31 HCL, 11 CLL, 4 NHL	41% CR, 15% PR	[151, 152]
HA22	CD22	PE	Ongoing		[33, 156]
A-dmDT390-bisFv(UCHT1)	CD22	DT	Ongoing		[65, 166]
DT2219	CD22, CD19	DT	Opening		[161]

Table 2.

Comparison of Phase I and II Trials of BL22 in HCL

	Phase I	Phase II	Difference
Patients enrolled	31	36	
Dose levels on protocol	2, 10, 20, 25, 30, 40, 50	30, 40	
Cycles administered	266	131	
Average dose/cycle	29	33	p = 0.39
Average cycles/patient	8.6	3.6	p < 0.0001
CR rate	61%	47%	p = 0.33
PR rate	19%	22%	p = 1.00
ORR	81%	69%	p = 0.40
Dose limiting toxicity from HUS	13%	6%	p = 0.40
Immunogenicity (% patients)	35%	11%	p = 0.021