Antibody Fusion Proteins: Anti-CD22 Recombinant Immunotoxin Moxetumomab Pasudotox

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Abstract

Recombinant immunotoxins are fusion proteins that contain the cytotoxic portion of a protein toxin fused to the Fv portion of an antibody. The Fv binds to an antigen on a target cell and brings the toxin into the cell interior, where it arrests protein synthesis and initiates the apoptotic cascade. Moxetumomab pasudotox, previously called HA22 or CAT-8015, is a recombinant immunotoxin composed of the Fv fragment of an anti-CD22 monoclonal antibody fused to a 38-kDa fragment of Pseudomonas exotoxin A, called PE38. Moxetumomab pasudotox is an improved, more active form of a predecessor recombinant immunotoxin, BL22 (also called CAT-3888), which produced complete remission in relapsed/refractory hairy cell leukemia (HCL), but it had a <20% response rate in chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL), diseases in which the leukemic cells contain much lower numbers of CD22 target sites. Compared with BL22, moxetumomab pasudotox is up to 50-fold more active on lymphoma cell lines and leukemic cells from patients with CLL and HCL. A phase I trial was recently completed in HCL patients, who achieved response rates similar to those obtained with BL22 but without dose-limiting toxicity. In addition to further testing in HCL, moxetumomab pasudotox is being evaluated in phase I trials in patients with CLL, B-cell lymphomas, and childhood ALL. Moreover, protein engineering is being used to increase its activity, decrease nonspecific side effects, and remove B-cell epitopes.

Introduction

Targeted toxins

Different approaches have been used and are under development to target toxic molecules to cancer cells and kill them more efficiently than would be possible with monoclonal antibodies (mAb) alone. Radioimmunotherapy is the most established of these approaches, represented by the approved drugs ibritumomab tiuxetan and tositumomab (1), and reviewed in this issue by Steiner and Neri (2). Exciting advances have been made with antibody-drug conjugates (3), particularly trastuzumab emtansine (4), SGN-35 (brentuximab vedotin; ref. 5), SAR3419 (6), and calicheamicin conjugates (7). Cytokines fused to antibodies are being used to attract immune effector cells to cancer cells (8). Immunotoxins are a type of antibody-conjugate in which a powerful toxin instead of a low-molecular-weight drug is attached to an antibody or antibody fragment. Their possible advantages include a very high activity, which enables them to kill cells with relatively few target sites, and a unique mechanism of action that can bypass some types of drug resistance. This review focuses on recombinant immunotoxins targeted to CD22, particularly moxetumomab pasudotox.

Protein toxins

Protein toxins produced by bacteria, fungi, and plants are extremely cytotoxic and can kill cells when only one or a few molecules reach the cytosol (9–12). These agents act by inhibiting protein synthesis and inducing apoptosis. The bacterial toxins Pseudomonas exotoxin A (PE) and diphtheria toxin (DT) catalytically inactivate elongation factor 2 by ADP ribosylation (9, 10). Plant toxins inactivate ribosomes by removing adenine in 28s ribosomal RNA (11, 13). Unlike plant toxins, bacterial toxins are made as single-chain proteins, making them more amenable for construction of recombinant chimeric proteins (14). Recombinant immunotoxins are produced by replacing the binding domain of the toxin with the Fv portion of an antibody that directs the toxin to a cancer cell. Target choice is very important to prevent killing of normal cells. The lineage-restricted differentiation B-cell antigen CD22 is an excellent target because it is absent on normal tissues like liver and skin, and its lack of expression on B-cell precursors allows normal CD22 B cells to be rapidly generated after immunotoxin therapy ceases.

CD22, a sialic acid–binding immunoglobulin-like lectin (siglec) that inhibits B-cell receptor calcium signaling, is expressed on many B-cell malignancies, including hairy cell
leukemia (HCL), chronic lymphocytic leukemia (CLL), B-cell non-Hodgkin lymphoma (NHL), and acute lymphoblastic leukemia (ALL; ref. 15). To kill these cells, we initially constructed an immunotoxin that reacts with CD22 by chemically conjugating a toxin fragment of PE to the LL2 antibody (16). In studies with this immunotoxin as well as several other chemical conjugates, we found that these conjugates were heterogeneous in composition and difficult to produce. Therefore, we turned to recombinant DNA techniques to make recombinant immunotoxins, which are stable, ~60- to 65-kDa, homogeneous chimeric proteins in which the Fv of a mAb is fused to truncated PE. The chimeric gene encoding the recombinant immunotoxin is introduced into *Escherichia coli*, where the recombinant protein is produced.

**Cellular intoxication by recombinant immunotoxins**

Crystallographic studies have shown that PE is made up of 3 major structural domains (Fig. 1). Domain Ia is the cell-binding domain, domain II contains a furin site that is necessary to release domain III from the cell-binding domain, and domain III contains the ADP-ribosylating activity that inactivates elongation factor 2. Domain Ib, which has no known function, can be partially or totally removed without affecting toxin activity. In recombinant immunotoxins targeting CD22, domain Ia of PE is removed and replaced by the Fv portion of an antibody reacting with CD22 as shown in Fig. 1.

For cancer treatment, the recombinant immunotoxin is administered i.v. so that it can reach all of the cells. After injection, the Fv at the amino terminus binds to CD22 present on the B-cell malignancy being targeted (17), and the carboxyl terminal lysine residue at position 613 (18) is rapidly removed by plasma carboxypeptidase, generating a protein ending in REDL. Very soon after binding, the recombinant immunotoxin-CD22 complex is internalized through clathrin-coated pits into the endocytic compartment (19), where 2 steps occur: (i) reduction of the disulfide bond in domain II, and (ii) a furin-catalyzed cleavage in the middle of domain II, resulting in the

![Figure 1. Intoxication of cells by moxetumomab pasudotox. Left, illustration of the structure of PE, PE38, and recombinant immunotoxin moxetumomab pasudotox designed to kill CD22-expressing cells. Right, cartoon showing the steps required for the entry and cell killing by moxetumomab pasudotox and similar recombinant immunotoxins containing PE38. After internalization, PE undergoes proteolysis and disulfide-bond reduction to separate the catalytic domain III from the binding domain Ia (20, 60, 61). PE38 undergoes both removal of the carboxyl terminal lysine residue (18) and processing between residues 279 and 280, resulting in a 37-kDa carboxyl terminal toxin fragment ending in the residues REDL. This fragment is believed to be transported intracellularly via the KDEL receptor from the Golgi to the endoplasmic reticulum (ER; 21), where it translocates to the cytosol, resulting in ADP-ribosylation of elongation factor 2 (9), leading to apoptotic cell death (25).](image-url)
separation of the Fv from domain III (Fig. 1; ref. 20). The REDL sequence at the carboxyl terminus of the protein then binds to the KDEL recycling receptor (21), and the ADP-ribosylating domain is transported to the endoplasmic reticulum, from which it translocates to the cytosol (22, 23). Once in the cytosol, the toxin catalyzes ADP ribosylation of the diphthamide residue (24) in EF-2 (9). This leads to a rapid decrease in the levels of the antiapoptotic protein Mcl-1 and, with the assistance of BAK, initiates the apoptotic cascade (25–29).

**Development of recombinant immunotoxins for CD22 malignancies**

The first recombinant immunotoxin that was designed to kill CD22-expressing cells contained a single-chain Fv of the anti-CD22 RFB4 antibody, which had been previously isolated by Peter Amlot in England, fused to PE38 (Fig. 1 and Fig. 2). This recombinant immunotoxin had relatively modest cytotoxic activity, with IC\textsubscript{50}s of 2 to 30 ng/mL, which we attributed to the instability of the 2 variable domains linked by the (G\textsubscript{4S})\textsubscript{3} peptide (30). To increase stability, we introduced cysteine residues into the light and heavy chains of the Fv so that a disulfide bond would form and stably anchor the light and heavy chains instead of the peptide linker that is used in most single-chain Fvs (Fig. 2; refs. 31–36). This protein was named BL22, for B-cell leukemia/lymphoma/CD22 (37).

**Development of BL22**

In preclinical studies, BL22 was cytotoxic to a wide variety of CD22-expressing cell lines, being 1.5- to 6.7-fold more cytotoxic than the single-chain recombinant immunotoxin (30, 37). BL22 induced complete remission (CR) in nude mice bearing CD22\textsuperscript{+} lymphoma xenografts at plasma levels that could be safely achieved in cynomolgus monkeys (38). Primary ALL, CLL, and NHL cells were also tested ex vivo and found to be sensitive to BL22 (39). CLL with as few as 350 CD22 sites/cell could be killed (39). Thus, the preclinical data justified the testing of BL22 in humans. A Good Manufacturing Practices lot of BL22 was produced at the Monoclonal Antibody and Recombinant Protein facility of the National Cancer Institute, and clinical trials were initiated in 1999.

**Phase I–II activity of BL22**

In the phase I trial, we elected to give BL22 every other day (QOD), for a total of 3 doses per cycle, and to repeat cycles every 21 to 28 days. The QOD dosing was based on animal experiments that showed that toxicity generally occurs within 48 hours of each dose. Therefore, keeping the dosing interval to 2 days in patients may prevent cumulative toxicity, particularly during dose escalation. In targeting HCL, where high CD22 density represents a sink for the drug, this dosing interval had an additional advantage in allowing dying cells to clear prior to the next dose. We began at 3 mg/kg and determined the maximum tolerated dose (MTD) to be 40 mg/kg /C2\textsuperscript{3}. In total, we treated 46 patients who had experienced failure of standard therapies for their disease, including 31 with HCL, 4 with B-NHL, and 11 with B-CLL (40, 41). In the 31 patients with HCL, 19 CRs (61%) and 6 partial remissions (PR; 19%) were achieved, for an overall response rate (ORR) of 81%. A hemolytic uremic syndrome composed of thrombocytopenia, hemolytic anemia, and renal insufficiency was observed in 4 HCL patients, and it completely resolved after 6 to 10 days of plasmapheresis. Dose-limiting capillary leak syndrome (CLS) was less common, appearing in only 1 HCL patient. Pharmacokinetic analysis of BL22 showed a strong sink effect of CD22.

![Figure 2. Recombinant immunotoxins in use or under development.](image-url)

BL22 was reported in 1997 (37) as RFB4(dsFv)-PE38 and was later called CAT-3888. VL and VH are disulfi
de-bonded together using engineered cysteine residues replacing Arg\textsuperscript{44} of VH and Gly\textsuperscript{100} of VL. In 2002, BL22 was mutated to HA22 (later called CAT-8015 and moxetumomab pasudotox) by changing SSY to THW at positions 100, 100a, and 100b of VH (45; horizontal red bars). In 2009, the deletion mutant HA22-LR was reported where PE amino acids 251–394 were replaced by amino acids 274–284 containing the Furin cleavage site (50). Most recently (in 2011), HA22-LR-8M, a mutant of HA22-LR, was reported to contain 8 mutations: D406A, R432G, R467A, R490A, R513A, E548A, K590S, and Q592A (59).
in HCL during the first cycle, caused by disease burden and high levels of soluble CD22, both of which decreased with response (42). Because 65% of CRs occurred after just 1 cycle of BL22, the phase II trial was planned to limit BL22 to 1 cycle and re-treat only those patients who had not achieved recovery of cytopenias to the level needed for CR. In 36 phase II patients receiving 1 cycle of BL22 at 40 μg/kg QOD × 3, there were 9 CRs (25%) and 9 PRs (25%), for an ORR of 50%. With selective re-treatment of 56% of the patients, the final ORR increased to 72%, including 47% CRs (43). The median time to relapse of cytopenias had not been reached after nearly 7 years of follow-up. This indicated significant activity in relapsed/refractory HCL with a safety profile supporting continued development. However, because the activity of BL22 was much lower in CLL, ALL, and NHL than in HCL (41, 44) and activity in the more common malignancies was essential for commercial development, we had to switch our clinical efforts to an improved immunotoxin.

**Development of moxetumomab pasudotox, an affinity-matured version of BL22**

An important variable in the activity of immunotoxins is their affinity for the target antigen, which determines how much immunotoxin will bind to a target cell at any given concentration and how long the cell-bound immunotoxin will remain on the cell surface. The longer it stays attached to the target protein (CD22), the more likely it is to enter and kill the target cell. BL22 has a moderate affinity for CD22 (Kd ~10 nmol/L). This affinity is sufficient to kill enough HCL cells in patients to achieve CR. HCL cells have a median of ~40,000 CD22 sites per cell, ranging from 10,000 to >100,000. However, BL22 was much less effective in children with ALL (4,500 sites/cell) or CLL (1,200 sites/cell). To improve the affinity and activity of BL22, we carried out mutagenesis studies and selected an Fv with a higher binding affinity by antibody phage display. Mutation of 3 residues in CDR3 of the heavy chain of the BL22 Fv (residues 100, 100a, and 100b) from SSY to THW increased affinity by ~15-fold and cytotoxicity toward HCL and CLL cells by up to 50-fold (45). The improvement in binding affinity was due to a slowing of the off rate, which led to increased cytotoxicity and antitumor activity despite similar pharmacokinetics and animal toxicity (46). The new, higher-affinity version of BL22 was initially named HA22 (high-activity BL22). Later, after it was licensed to Cambridge Antibody Technologies, it was named CAT-8015, and it is now being developed by MedImmune as moxetumomab pasudotox.

**Phase I testing of moxetumomab pasudotox**

At the 2010 American Society of Hematology Annual Meeting (Orlando, FL; December 4–7, 2010), interim results were reported for clinical trials with moxetumomab pasudotox in HCL and ALL. Clinical trials are listed in Table 1. Several striking differences between moxetumomab pasudotox and BL22 have been noted. One is that in the HCL trial, dose-limiting toxicity was not observed and dose escalation was terminated at 50 μg/kg, a dose level above the MTD for BL22, because response rates were high at all dose levels. In fact, the ORR from an interim analysis of moxetumomab pasudotox, 79% (47), was similar to the 72% ORR reported from the phase II study of BL22 (43). Another striking finding is that 3 responses (all CRs) were observed in 12 patients with pediatric ALL (48), representing the first time responses of this magnitude were observed with this type of agent in this aggressive disease.

**Interim phase I results in HCL**

At the 2010 American Society of Hematology Annual Meeting (49), investigators presented findings obtained from 32 patients with refractory HCL, all of whom had received 2 or more prior courses of purine analogue therapy and needed treatment based on established criteria for HCL (i.e., neutrophils <1000/mm³, platelets <100,000/mm³, hemoglobin <10 g/dl, or symptomatic splenomegaly). Patients received dose levels of 5 to 50 μg/kg QOD × 3 in a standard dose-escalation phase, with cycles repeated every 4 weeks. Patients were re-treated until they reached 2 cycles past CR; however, treatment was stopped earlier if progressive disease or immunogenicity was detected. Surprisingly,

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<th>Table 1. Ongoing phase I trials with moxetumomab pasudotox</th>
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Abbreviations: CCCN, Comprehensive Cancer Center of Nevada; CSMC, Cedars Sinai Medical Center; DF, Dana-Farber Cancer Institute; DLBCL, diffuse large B-cell lymphoma; HSCT, hematopoietic stem cell transplant; IU, Indiana University; MCL, mantle cell lymphoma; MUSC, Medical University of South Carolina; SCRI, Sarah Cannon Research Institute; SJ, Saint Jude Children’s Research Hospital.

<sup>a</sup> Listed as a phase I-II trial.
no dose-limiting toxicity was observed in the 32 patients, despite the fact that half of the patients enrolled \((n = 16)\) were at the 50 \(\mu\)g/kg \(\times\) 3 dose level, which was higher than the MTD of BL22 \((41)\). The most common adverse events, seen in 20% to 50% of patients, were expected from prior experience with immunotoxins. These included mild CLS causing weight gain, hypoalbuminemia, peripheral edema, fever, elevated transaminases, fatigue, and headache. The only evidence of possible hemolytic uremic syndrome was asymptomatic laboratory abnormalities in 2 patients, with peak creatinine of 1.53 to 1.66 mg/dL and platelet nadir of 111,000 to 120,000/mm\(^3\). Major responses were observed at all dose levels in the 32 patients reported, with CRs observed at all dose levels from 10 to 50 \(\mu\)g/kg QOD \(\times\) 3, and the CR rate at all doses was 31% at the time of interim analysis. The median time to response was 2.8 months \((49)\). Only 1 of the 14 patients who achieved CR relapsed in less than 1 year, indicating...
that responses were durable. In summary, moxetumomab pasudotox showed high and durable antitumor activity in patients with relapsed/refractory HCL, statistically similar to BL22 but without dose-limiting toxicity, justifying further clinical development toward the goal of approval for this disease. Moxetumomab pasudotox is also being evaluated in other trials, but data are not yet available from those trials.

**Interim phase I results in ALL**

Because pediatric ALL is a much more rapidly growing and aggressive disease than HCL, patients were treated with moxetumomab pasudotox QOD for 6 doses, and the cycles were repeated every 21 days (48). A rapid dose-escalation scheme was used for the low dose levels (5, 10, and 20 μg/kg QOD × 6), and then a standard dose escalation began at 30 μg/kg QOD × 6. Fourteen patients 5 to 22 years of age (median, 11 years) were enrolled in the trial. All of these patients had been heavily pretreated with 2 to 8 (median 4) prior regimens, and 7 had previously undergone stem cell transplantation. Common toxicities included elevated bilirubin, transaminases, and creatinine, and CLS including hypalbuminemia, proteinuria, hypoxia, and pleural effusion. Grade 3/4 CLS was observed in 2 of the first 7 patients (both treated at 30 μg/kg QOD × 3) but not in 7 subsequent patients once dexamethasone prophylaxis was instituted. As mentioned above, of 12 patients who were evaluable for response, 3 (25%) achieved CR after 1 to 2 cycles, and 5 (42%) had hematologic improvement with reduction in circulating blasts. As in the HCL trial, CRs were observed at dose levels as low as 10 μg/kg. Thus, moxetumomab pasudotox has achieved major responses, including CRs, in patients with ALL. Due to the aggressive nature of the disease and the young age of these patients, even a transient CR can be lifesaving as a bridge to transplantation. Further clinical development of moxetumomab pasudotox is proceeding in ALL to determine whether higher dose levels and more frequent dosing can improve responses.

**A Protease-Resistant Moxetumomab Pasudotox**

One impediment to the cytotoxic activity of immunotoxins is that many of the immunotoxin molecules that enter target cells do not reach the endoplasmic reticulum and cytosol; instead, they are transferred to lysosomes and other compartments containing lysosomal enzymes, where they are inactivated by proteolysis (Fig. 1). To identify potential lysosomal protease cleavage sites, investigators treated recombinant immunotoxins with lysosomal enzymes and identified the cleavage sites by amino acid analysis (50). These studies revealed that all of the major protease sites were clustered in domain II and could be removed, leading to a new lysosomal protease-resistant immunotoxin named HA22-LR (Fig. 2 and Fig. 3). In HA22-LR, all of domain II is deleted except for the 11 amino acids that make up the essential furin-processing site. HA22-LR had several useful properties, including increased activity, particularly on CLL cells, where HA22-LR had a median 16-fold improved cytotoxic activity compared with HA22 and more than 10-fold reduced nonspecific toxicity to mice (50). The latter finding may be due to loss of residues that interact with endothelium or macrophages and produce liver damage in mice and possibly CLS in other animals, including humans (50–53). It is possible that the smaller size of HA22-LR (51.0 kDa versus 63.3 kDa for HA22) may allow improved tumor penetration. Finally, because we could safely give much higher doses to mice, we were able to achieve much better antitumor activity (50).

**A Moxetumomab Pasudotox Variant with No Immunogenicity in Mice**

Although the incidence of antibody formation in patients treated with moxetumomab pasudotox is low and did not prevent CRs in HCL and ALL, further decreases in toxin immunogenicity remain an important goal. For patients with solid tumors and normal immune systems, immunogenicity rates of 80% to 90% were observed after 1 cycle of 3 doses (54–56). To deimmunize the toxin, efforts to identify and remove both T-cell (57) and B-cell (58) epitopes are underway. To date, more progress has been made with the B-cell epitope approach (53, 59). On the basis of the hypothesis that human and mouse B-cell epitopes are similar, we used mice for these studies and identified 7 major epitopes in PE38: 3 in domain II, and 4 in domain III. The epitopes in domain II are removed in HA22-LR (50), which has a deletion of most of domain II, and the epitopes in domain III were destroyed by mutating 8 different bulky amino acids to alanine, serine, or glycine to produce HA22-LR-8M (59). We measured the reactivity of HA22-LR-8M with serum from patients who made antibodies to HA22, and we found that reactivity with antisera was greatly decreased but not abolished. We are now engaged in studies to identify and remove the remaining human-specific epitopes. Our data suggest that new immunotoxins with low immunogenicity will not be needed for the successful treatment of B-cell malignancies, because the immune system is very suppressed. However, they will be useful for the treatment of solid tumors, where neutralizing antibodies develop in the vast majority of patients after 1 cycle of 3 doses. For HCL in particular, we believe the clinical results (both efficacy and safety profile) for moxetumomab pasudotox justify further clinical development, including a pivotal phase III trial currently under design, with the goal of achieving U.S. Food and Drug Administration approval for the use of moxetumomab pasudotox for this disease.

**Disclosure of Potential Conflicts of Interest**

I. Pastan is an inventor on patents on moxetumomab pasudotox held by the NIH. The authors disclose no other potential conflicts of interest.
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