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Immunotoxins targeting CD22

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CONTENTS

Abstract

CAT-3888 (RFB4[dsFv]-PE38, BL22) is a recombinant immunotoxin consisting of disulfide bond-linked Fv fragments of the parental anti-CD22 monoclonal antibody RFB4 fused to PE38, a truncated form of the bacterial *Pseudomonas* exotoxin A. Following binding to CD22, this immunotoxin is internalized into the target cell by endocytosis, where it is processed to generate a free toxin fragment which, after translocation into the cytoplasm, induces cell death by catalytic inactivation of elongation factor 2 (EF-2). Since many B-cell malignancies express the CD22 antigen on their cell surface, CAT-3888 is intended to treat patients with such cancers. Indeed, initial clinical evaluation demonstrated efficacy in the treatment of chemoresistant hairy cell leukemia.

Background

Despite standard therapies of surgery, chemotherapy and radiation, cancer remains one of the most life-threatening diseases. One reason for the failure of most therapies can be ascribed to the fact that it is difficult to differentiate tumor cells from healthy cells, and thus most therapies cause severe side effects. Indeed, despite intensive research on cancer and cancer therapy over the past decades, the prognosis for metastatic cancer has not been improved satisfactorily. Nevertheless, many characteristics of different tumor entities, especially concerning the signaling pathways and molecular aberrations involved in carcinogenesis, have been unraveled. Based on this information, specific tumor therapies have been established which either target the proteins involved in the neoplastic process directly, or specifically target toxic drugs to the tumor. Importantly, monoclonal antibodies (MAbs) can be used to realize both strategies because of their attributes of specificity and high affinity. In this regard, conjugates of tumor-specific MAbs with radionuclides, cytotoxic drugs, toxins or cytokines have been evaluated (1). This strategy should increase the specificity of the therapeutic intervention and thereby minimize side effects while maximizing the desired effects.

Immunotoxins are proteins that contain a toxin, *i.e.,* a poisonous substance produced by living cells or organisms, along with an antibody or peptide that binds specifically to target cells. Indeed, toxins or toxin subunits derived from microorganisms, plants, insects and vertebrates, such as diphtheria toxin, ricin, golin, saporin, pokeweed and *Pseudomonas* exotoxin A (PE), have been conjugated to MAbs and tested for antitumor efficacy (2). Toxins are enzymes that exert their cytotoxic activity inside the cell, mostly by inhibiting protein synthesis enzymatically, and in most cases one single molecule is sufficient to kill the cell. To be used therapeutically, toxins have to be modified to remove their tissue binding sites (3). Furthermore, deglycosylation of toxins prevents their rapid clearance by liver cells expressing mannose receptors. The affinity of immunotoxins for an antigen determines the quantity and quality of association with the cell-surface antigen. Notably, an increased affinity is generally associated with a higher killing capacity of the immunotoxin. However, high local concentrations of immunotoxin may allow nonadsorptive or pinocytic uptake into nontarget cells, causing undesirable side effects. Besides antigen binding affinity, internalization rate, intracellular processing and the intrinsic potency of the toxin domain determine the cytotoxic potency of immunotoxins (4). Importantly, toxicities are not as high as would be concluded from the toxicity of the toxin *per se*, as the uptake of the immunotoxins into the intracellular compartment is often rather inefficient.

Because antigens and receptors found on the surface of B-cell neoplasms are frequently readily accessible from plasma, they represent attractive targets for immunotoxin therapy. CD22, belonging to the sialic acid-binding immunglobulin-like lectin (SIGLEC) family (5), is an example of an antigen expressed on B-cells, where it functions as an inhibitory receptor for B-cell receptor signaling (6). CD22 thereby prevents the overactivation of the immune response and the development of autoim-

mune diseases (7). CD22 expression is observed at low levels in the cytoplasm of pro- and pre-B-cells, and at high levels on the cell surface of mature cells with IgM and IgD positivity (8). On germinal center B-cells, the presence of CD22 is limited, whereas it is more strongly expressed on the surface of follicular, mantle and marginal zone B-cells. Moreover, CD22 expression is frequently preserved in B-cell-derived malignancies (9). Indeed, CD22 expression is found in approximately 80% of non-Hodgkin's lymphomas (NHL), more than 90% of pediatric acute lymphoblastic leukemias (ALL) and in virtually all B-cell chronic lymphocytic leukemias (CLL) and hairy cell leukemias (HCL) (10).

NHL is the fifth most common cancer in the U.S. The age-adjusted incidence of NHL rose by 84% from 1975 to 2004 and about 63,190 people in the U.S. are expected to be diagnosed with NHL in 2007 (http://www.leukemia-lymphoma.org). ALL is the most common cancer diagnosed in children and represents 25% of cancer diagnoses among children younger than 15 years (http://seer.cancer.gov/publications/childhood/leukemia.pdf). In the U.S., approximately 2,400 children and adolescents younger than 20 years are diagnosed with ALL each year (http://seer.cancer.gov/publications/childhood/leukemia.pdf) and there has been a gradual increase in the incidence of ALL in the past 25 years (11). In contrast to ALL, CLL is a disease of elderly people. More than three-fourths of those who suffer from CLL are over 60 years of age (http://www.merck.com/mmhe/sec14/ch176/ch176d.html). In 95% of cases, CLL is a disorder of B-lymphocytes. About 15,340 new cases of CLL will be diagnosed in the U.S. in 2007 (http://www.leukemia-lymphoma.org). In contrast, HCL is a rather rare B-cell malignancy with only 500-800 new cases per year, comprising about 2% of all leukemias (12). Patients are characterized by pancytopenia and variable infiltration of the reticuloendothelial system with hairy lymphocytes, *i.e.,* malignant cells with eccentric kidney-shaped spongiform nuclei and hair-like cytoplasmic projections. The standard treatment of the aforementioned cancers includes chemo- and radiotherapy, and MAbs have been added to the available treatment options in the past years (13-15). However, the death rates are still high, indicating the need for further improvement of therapy (16).

In this regard, several immunotoxins targeting CD22 have already been generated. Fused toxins include plant toxins such as saporin, gelonin, ricin A chain and pokeweed antiviral protein and the bacterial toxin PE (17-20). The latter is especially attractive, since linkage to antibodies can be achieved by disulfide, thioether and peptide bonds. An earlier immunotoxin consisting of the anti-CD22 MAb RFB4 and ricin A caused vascular leakage syndrome. However, since it was demonstrated that this toxicity was related only to ricin, the hope was that the use of PE might prevent this side effect. Mansfield *et al.* generated different immunotoxins consisting of the murine anti-CD22 MAb RFB4 and truncated versions of PE (21). The covalently coupled conjugates were effective in experimental lymphoma xenograft models and displayed little cytotoxicity towards human vascular endothelial cells *in vitro*. However, they are relatively large and often heterogeneous at their linkage site, which may result in suboptimal activity. In addition, the ability to penetrate tumor masses might be impaired for whole antibody-toxin conjugates, as the ability to penetrate tumors is inversely related to the size of the penetrating molecule. Consequently, the same group cloned a disulfide-linked (ds) Fv fragment of RFB4 fused with PE38, which results in a marked reduction of size compared to chemical IgG-toxin conjugates. This RFB4(dsFv)-PE38 (BL22, CAT-3888) immunotoxin was tested preclinically and clinically for the treatment of B-cell malignancies.

CAT-3888 functions by binding of the anti-CD22 moiety to CD22, upon which the immunotoxin is internalized into an acidic endosome (Fig. 1). There, domain II is cleaved by the protease furin. After the reduction of a certain disulfide bond, two proteolytic fragments are released. The *C*-terminal fragment is then transported from the endocytic compartment or the transreticular Golgi apparatus to the endoplasmic reticulum. Following translocation to the cytosol, the toxin will ribosylate elongation factor 2 (EF-2) and mediate cell death by apoptotic or nonapoptotic mechanisms (22).

Preparation of CAT-3888

Full-length PE consists of 613 amino acids and can be divided into 3 major functional domains: domain I is responsible for binding to the PE receptor present on most cells, domain II mediates the translocation across membranes and domain III exerts the toxic function by ADB ribosylation of EF-2 (Fig. 2). PE38 is a truncated form containing domains II and III and only part of domain I lacking the binding domain. PE38 is genetically fused to the cloned V_H fragment of the MAb RFB4. Notably, the cloned V_L and V_H fragments each contain a cysteine residue replacing Gly100 and Arg44, respectively, allowing the formation of a disulfide bond connecting V_L and V_H. The expression plasmids encoding V_L- and V_H-PE38 are separately expressed in *Escherichia coli*. After the induction of protein production, the components of the immunotoxin accumulate in insoluble bacterial inclusion bodies. Subsequently, these inclusion bodies are washed extensively with detergent to remove endotoxin before they are solubilized and reduced by treatment with guanidine-dithioerythritol solution. Equal weight amounts of V_L- and V_H-PE38 are used to re-fold CAT-3888. Disulfide-linked immunotoxin is allowed to re-fold for 48 h. Finally, dialyzed re-natured protein is purified by anion exchange and size exclusion chromatography.

Preclinical pharmacology, pharmacokinetics and safety of CAT-3888

In vitro, CAT-3888 demonstrated cytotoxicity against different Burkitt's lymphoma cell lines, while being inactive against a CD22-negative T-cell line. IC_{50} values ranged from 0.25 to 20 ng/ml. Noteworthy, an IC_{50} of 20

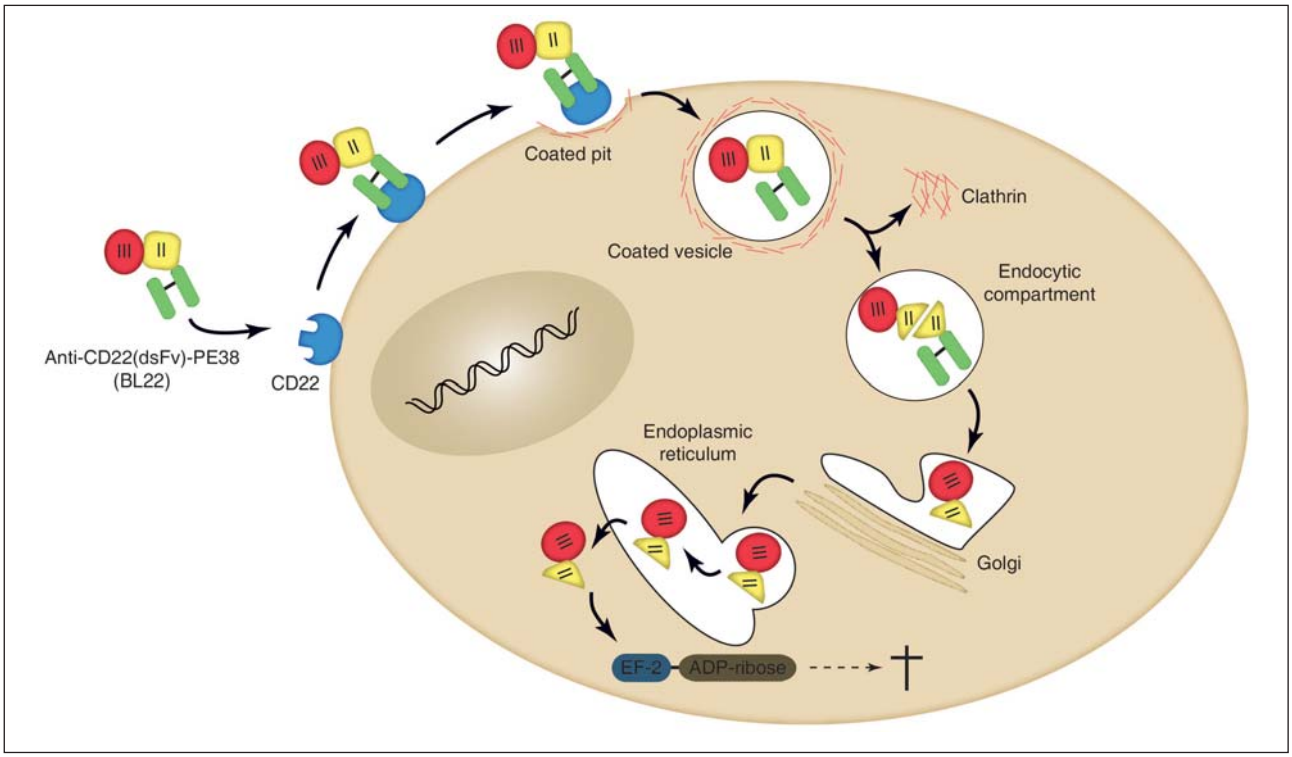

Fig. 1. Killing of CD22-expressing cells by CAT-3888 (BL22). The immunotoxin enters the cells after binding to CD22 by endocytosis. In the cell, CAT-3888 is processed and translocated through the endoplasmic reticulum to the cytosol, where it ADP-ribosylates elongation factor 2 (EF-2), causing cell death.

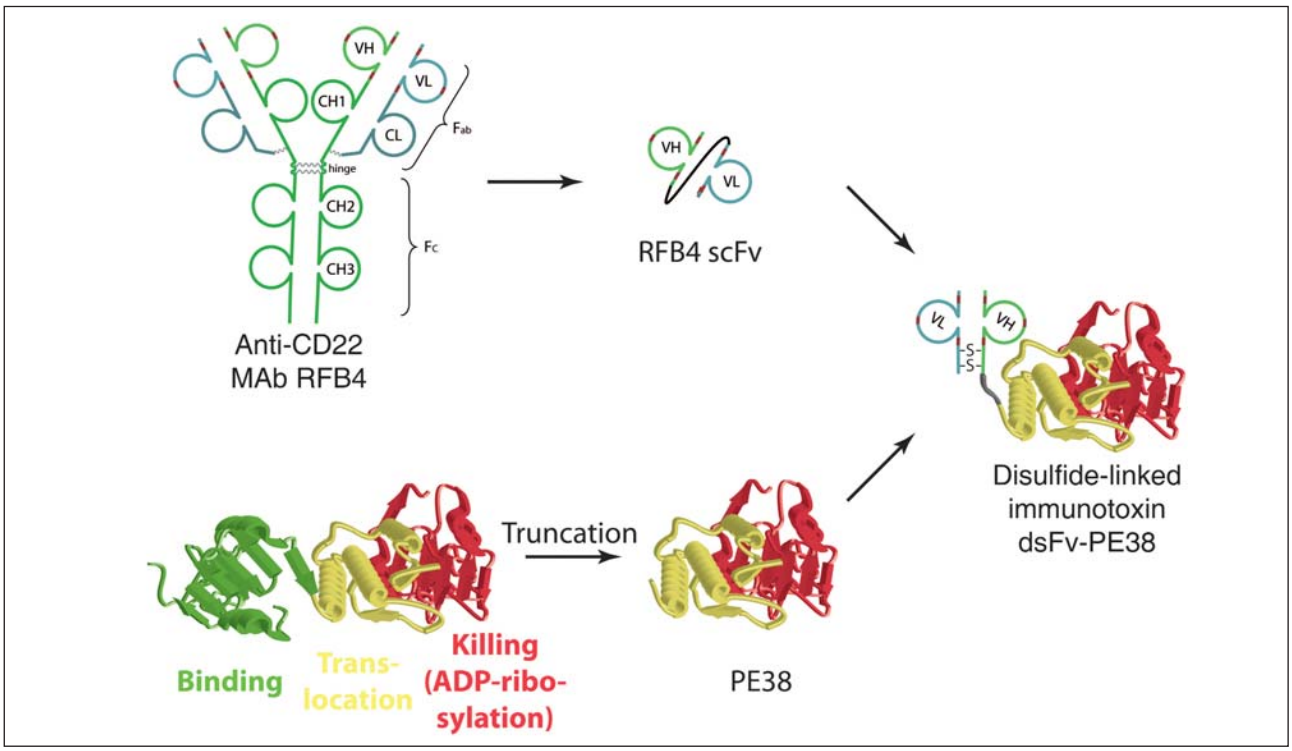

Fig. 2. Schematic structure of CAT-3888 and *Pseudomonas* exotoxin (PE). PE contains 3 functional domains, *i.e.,* a binding domain, a translocating domain and a killing domain, which as an ADP-ribosylating enzyme inactivates elongation factor 2 (EF-2). PE38 is a fragment of PE lacking the binding domain. The recombinant disulfide-stabilized immunotoxin RFB4(dsFv)-PE38 (CAT-3888, BL22) is comprised of a V_L disulfide bonded to a fusion of V_H with PE38.

ng/ml was observed against Daudi cells, which cannot efficiently proteolytically process PE. The binding capacity of the highly stable CAT-3888 was comparable to the native RFB4 IgG. In addition, *in vitro* assays revealed that cell lines required exposure for > 2 h to bind and internalize maximal amounts of the immunotoxin, with complete intoxication after 24 h. In initial experiments, the maximum tolerated dose (MTD) in mice was 7 µg when given i.v. every other day for three doses. When administered as several daily doses, the immunotoxin exerted an antitumor effect in a xenogeneic model, *i.e.,* partial responses were apparent in irradiated nude mice bearing human CA46 tumors (23).

By slight modifications of the procedure, the immunotoxin was produced on a larger scale, with greater purity and reduced toxicity, and was further evaluated in preclinical models. The LD_{50} was 1025 µg/kg when given i.v. x 3 on alternate days (QOD x 3). Following continuous i.p. infusion, the LD_{50} was 1040 µg/kg/day. Since the RFB4 MAb binds to primate but not murine CD22, the toxicity of CAT-3888 was also tested in cynomolgus monkeys. Doses of 100 or 500 µg/kg QOD x 3 were well tolerated, with only mild laboratory abnormalities. For example, there was a drop in hemoglobin and albumin after treatment. The only hepatic abnormalities were borderline elevated alanine aminotransferase (ALT), alkaline phosphatase and lactate dehydrogenase (LDH). In subsequent studies conducted by the National Cancer Institute (NCI), 2000 µg/kg could be administered to monkeys without serious toxicity (22).

In the above tumor model of s.c.-injected CA46 cells in nude mice, treatment with CAT-3888 at a dose of 350 or 275 µg/kg i.v. QOD x 3 resulted in complete remissions (CRs) in all mice. At the dose of 200 µg/kg, 60% of the mice had CR. When mice were treated by continuous infusion, all mice treated with 100 or 200 µg/kg/day had CR while mice treated with 50 µg/kg/day displayed only a slight delay in tumor growth compared to control mice (22).

Plasma levels of CAT-3888 were determined in nude mice after dosing i.v. QOD x 3 or by continuuous i.p. infusion. The immunotoxin was quantified by a cytotoxicity assay against a CD22-positive Burkitt's lymphoma cell line to obtain data on active toxin with an intact and functional binding domain. After an i.v. bolus of 200 µg/kg of CAT-3888, plasma levels reached 4200 ± 880 ng/ml. Plasma levels decreased biexponentially, with a $t_{1/2\alpha}$ of 30 min and a $t_{1/2\beta}$ of 290 min. The majority of plasma clearance, *i.e.,* more than 99%, occurred in the α-phase. The natural log of plasma concentration *versus* time was 0.022/min. For the 200 µg/kg dose, the AUC was 180 µg.min/ml. Following continuous infusion of 200 µg/kg/day for 7 days, mean plasma concentrations ranged from 95 to 125 ng/ml on days 2-5 and at day 7 the concentration was still 100 ng/ml. The AUC for this scheme was 950 µg.min/ml (22).

In cynomolgus monkeys, peak levels of the immunotoxin after 2 min were 3.1 µg/ml for the dose of 100 µg/kg and 14 µg/ml for the dose of 500 µg/kg. In both cases, concentrations decreased monoexponentially, with half-lives of 66 and 44 min, respectively. The AUC was 280 µg.min/ml for the 100 µg/kg dose and 870 µg.min/ml for the 500 µg/kg dose. Thus, the ratio of the AUC to the dose per kg is higher in monkeys than in mice (22).

In the clinical setting, peak plasma levels were dose-related and the median half-life was about 3 h at doses of 30-50 µg/kg QOD x 3. The AUC was significantly correlated with dose. Moreover, due to the high level of CD22 expression on malignant cells, high variability was observed. Indeed, a reduction in tumor burden translated to increased AUC values and peak plasma levels. In addition, the volume of distribution fell to the level of the plasma volume. Consequently, re-treatments were performed at lower doses than the initial dose. The MTD evaluated at cycle 1 was 40 µg/kg i.v. QOD x 3 (24).

Clinical evaluation of CAT-3888

In order to determine the safety and tolerability of CAT-3888, a phase I clinical study was conducted in 46 pretreated patients with $CD22^+$ B-cell malignancies (24, 25). The patient cohort included 4 with NHL, 11 with CLL and 31 with HCL. A total of 265 cycles were administered at 3-50 µg/kg QOD x 3, and 35 patients received more than 1 cycle. The most common dose-limiting toxicity (DLT) was hemolytic uremic syndrome (HUS), observed in 5 patients. Four of these patients completely recovered after plasmapheresis. In the remaining patient, HUS could not be assessed due to death caused by progressive NHL. The mechanism of HUS development upon CAT-3888 therapy has not yet been resolved. Since ADAMTS-13 (a disintegrin and metalloproteinase with thrombospondin motifs 13) function was normal, the circulation of high levels of unusually long multimers of von Willebrand factor can be excluded as a causative factor. Therefore, it is suggested that HUS development does not require plasma exchange (10). Notably, other PE-containing recombinant immunotoxins do not elicit HUS, indicating that the mechanism of HUS can be somehow ascribed to the CD22-binding domain. As the only other DLT, a cytokine release syndrome with systemic vascular leak syndrome was observed in 1 patient. Since no DLT was observed in 12 patients treated with 40 µg/kg QOD x 3 during cycle 1, this dose level was considered to be the MTD of cycle 1.

CAT-3888 was well tolerated at the MTD during cycle 1. Indeed, only grade 1-2 reversible toxicities were observed, of which fatigue, myalgia, ALT and aspartate aminotransferase (AST) elevations and hypoalbuminemia were most common, *i.e.,* each affecting at least half of the patients. Less common toxicities included fever and low-grade vascular leak syndrome. Notably, CAT-3888 did not adversely affect the CD4 cell count, which is often observed upon treatment with purine analogues, the standard therapy for HCL (26). The immunogenicity of CAT-3888 was determined by a bioassay to detect neutralizing antibodies. The cytotoxic activity against a $CD22^+$ Burkitt's lymphoma cell line was measured after incubation of purified CAT-3888 with patient serum.

Eleven patients presented with neutralizing antibodies after 1-8 cycles of therapy. Notably, the development of such antibodies occurred only in HCL patients, but was not dose-related.

The majority of patients included in the phase I trial had HCL, and these 31 patients had been extensively pretreated. It should be noted that HCL patients are on the one hand highly responsive to available therapy, but on the other hand can not be cured by these therapies. Indeed, a CR rate of > 79-95% to either cladribine or pentostatin has been observed and these patients remain in CR at median follow-up times of 9-15 years (26, 27). Furthermore, 50-70% of patients achieve CR on a second course of therapy. However, disease-free survival curves, as well as molecular biology, demonstrate that these therapies are not curative, indicating the need for alternative HCL therapies. Patients enrolled in the CAT-3888 clinical trial had to have a CR for < 2 years after the last course of cladribine, but many of the patients demonstrated a very poor response to the previous therapy. Nonetheless, the response to CAT-3888 in these patients was very high: CR in 19 patients (61%) and PR in 6 patients (19%). All responders achieved significant improvement in cytopenia. Eleven of the CRs occurred after only 1 cycle, while the rest required 2-14 cycles, and the CR rate was dose-related. Moreover, 5 CRs and 2 PRs were documented for the 11 patients with neutralizing antibodies. Interestingly, 10 of the 19 patients with CRs had never achieved CR to any prior therapy. In addition, 6 of the 9 remaining CRs did not have a response to the last course of purine analogue. The median duration of CR was 36 (range: 5-74) months. Seven of 19 CRs were still in CR at a median of 54 (range: 36-74) months (10). In summary, patients with chemoresistant HCL had a high response rate to CAT-3888, including patients who demonstrated a lack of response to purine analogue therapy (24). Patients with a high tumor burdon may require extra cycles for optimal response. Currently, a phase II trial is recruiting patients to determine the response rate in patients with cladribine-resistant HCL treated with CAT-3888 (28).

Future directions

In the phase I study of CAT-3888, only a few patients with CLL or NHL were enrolled. These patients, however, did not respond as well as patients with HCL, even at doses of 30-50 µg/kg QOD x 3. Only minor and marginal responses could be documented. A limiting factor in treating CLL with CAT-3888 is the low expression of CD22. Thus, to increase the amount of toxin internalized by the target cell, the affinity of CAT-3888 was improved by mutagenesis of the complementarity-determining region 3 of the heavy chain of the Fv fragment. The resulting engineered immunotoxin, CAT-8015 (HA22), in which the amino acids at positions 100, 100a and 100b were replaced, has > 10-fold improved cytotoxicity toward CLL cells compared to CAT-3888. CAT-8015 is also more cytotoxic to cells from patients with HCL (29, 30). Currently, three phase I trials are recruiting patients to evaluate CAT-8015 in patients with HCL (31), CLL, prolymphocytic leukemia or small lymphocytic lymphoma (32), or NHL (33); a total of 150 patients will be enrolled.

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