Anti-CD22 Immunotoxin RFB4(dsFv)-PE38 (BL22) for CD22-Positive Hematologic Malignancies of Childhood: Preclinical Studies and Phase I Clinical Trial

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Abstract

Purpose: Although most children with B-lineage acute lymphoblastic leukemia (ALL) and non–Hodgkin lymphoma are cured, new agents are needed to overcome drug resistance and reduce toxicities of chemotherapy. We hypothesized that the novel anti-CD22 immunotoxin, RFB4(dsFv)-PE38 (BL22, CAT-3888), would be active and have limited nonspecific side effects in children with CD22-expressing hematologic malignancies. We conducted the first preclinical and phase I clinical studies of BL22 in that setting.

Experimental Design: Lymphoblasts from children with B-lineage ALL were assessed for CD22 expression by flow cytometry and for BL22 sensitivity by in vitro cytotoxicity assay. BL22 was evaluated in a human ALL murine xenograft model. A phase I clinical trial was conducted for pediatric subjects with CD22+ ALL and non–Hodgkin lymphoma.

Results: All samples screened were CD22+. BL22 was cytotoxic to blasts in vitro (median IC50, 9.8 ng/mL) and prolonged the leukemia-free survival of murine xenografts. Phase I trial cohorts were treated at escalating doses and schedules ranging from 10 to 40 μg/kg every other day for three or six doses repeated every 21 or 28 days. Treatment was associated with an acceptable safety profile, adverse events were rapidly reversible, and no maximum tolerated dose was defined. Pharmacokinetics were influenced by disease burden consistent with rapid drug binding by CD22+ blasts. Although no responses were observed, transient clinical activity was seen in most subjects.

Conclusions: CD22 represents an excellent target and anti-CD22 immunotoxins offer therapeutic promise in B-lineage hematologic malignancies of childhood.
and phase I clinical trial of BL22 for pediatric ALL and non–Hodgkin lymphoma.

Materials and Methods

Patient samples. Fresh bone marrow or peripheral blood blasts were collected from children with B-lineage ALL.

In vitro cytotoxicity. Seventy-two–hour in vitro cytotoxicity assays were conducted using protein synthesis inhibition ([3H]leucine incorporation) and colorimetric viability (WST-1). Results were expressed as the IC50 value (concentration of BL22 required to reduce viability/protein synthesis by 50% compared with nontreated controls) as previously described (12).

Flow cytometry and antigen binding site determination. CD22 antigen expression and absolute peripheral blast counts were determined by flow cytometry. Antigen site density was quantified by determining the anti-CD22 antibody binding capacity per cell (17) using the BD Biosciences QuantiBRITE system for fluorescence quantitation.

Murine xenografts. Cells from the human ALL line EU-1 were used for xenograft studies. This cell line was established and authenticated as previously described (18), and phenotype was reconfirmed by serial flow cytometric analyses including at the time of the xenograft studies. EU-1 cells were injected by tail vein into 5-wk-old female C.B-17 severe combined immunodeficient mice−/− (10⁷ cells per mouse). Seventy-two hours after injection, cohorts of 10 (treatment) or 5 (control) xenografts were treated with BL22 at dose levels of 1.5, 3, or 4.5 μg/dose, or control agents through i.p. injection every other day for nine doses. Xenograft recipients were euthanized at hind-limb paralysis and evaluated for the presence of human leukemia by histopathology.

BL22 and control agents. Recombinant immunotoxins were produced as previously described (11, 12, 19). Clinical grade BL22 for human use was produced by the Monoclonal Antibody and Recombinant Protein Facility of the National Cancer Institute (NCI) and provided by MedImmune Cambridge (formerly Cambridge Antibody Technology, Inc., a subsidiary of MedImmune, LLC). Control reagents for preclinical studies included PBS, anti–CD22 MoAb (RF84-IgG provided by the Developmental Therapeutics Program of the NCI), and the anti–CD25-PE immunotoxin anti–Tac(Fv)-PE38 (LMB2).

Phase I clinical trial. A phase I trial was conducted at the NIH Clinical Center (ClinicalTrials.gov no. NCT00077493).

Eligibility. Individuals between 6 mo and 24 y of age with relapsed or refractory ALL or non–Hodgkin lymphoma who had exhausted available curative therapies were eligible. Measurable or evaluable disease burden. Eligibility required aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of five or more times the upper limit of the normal, total bilirubin of ≤2 mg/dL, and age-adjusted normal creatinine.

BL22 administration. BL22 was administered i.v. over 30 min every other day for three or six doses. Intravenous hydration was initiated 6 h before BL22 using 5% dextrose and 0.45% sodium chloride at a rate of 90 mL/m²/h. Pre-medication consisted of acetaminophen, diphenhydramine, and ranitidine.

Study design. Cohorts of three to six subjects were treated at doses of 10, 20, 25, 30, and 40 μg/kg/dose. During the course of the trial, the escalation scheme was modified to shorten the treatment interval from 28 to 21 d and to increase the number of doses per cycle from three to six (Table 1). If dose-limiting toxicity (DLT) was encountered in one subject in a cohort of three, an additional three patients were entered at that dose level. If DLT occurred in two or more patients at any given dose level, the maximum tolerated dose was considered to have been exceeded. Re-treatment required the recovery of BL22-related toxicity to lower than grade 2 and the absence of DLT, high-tier neutralizing antibodies, and disease progression.
Toxicity grading and definition. Version 3.0 of the NCI Common Terminology Criteria for Adverse Events\textsuperscript{6} was used for toxicity and adverse event reporting. DLT was defined as a nonhematologic toxicity of grade 3 or more with the following exceptions: tumor lysis syndrome, abnormal electrolytes responding to supplementation, grade 3 hepatic dysfunction with resolution before the next cycle, and grade 3 fever, hypertriglyceridemia, hypercholesterolemia, and hypoalbuminemia in the absence of vascular leak syndrome. Hematologic DLT was defined as grade 4 hematologic toxicity (with the exception of lymphocytes)

\begin{table}
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\begin{tabular}{lllllll}
\hline
ID & Dx & Age (y) & Disease status & Steroids & CD22 & CD22 site density & PB blast count Pre/peak Post/nadir

\hline
1 & ALL & 9 & Relapse no. 5, refractory, nine prior regimens & 100 & 6,478 & 43 & 17
2 & ALL/ LBL & 14 & Relapse no. 6, refractory, eight prior regimens, allo-SCT, extramedullary chloromas & 100 & 1,661 & 0 & 0
3 & ALL & 13 & Relapse no. 1, refractory, seven prior regimens & + & 100 & 4,341 & 2,401 & 3,160
4 & ALL & 22 & Relapse no. 1, refractory, four prior regimens & 100 & 2,303 & 96,660 & 69,540
5 & ALL & 19 & Relapse no. 1, refractory, six prior regimens & 100 & 1,835 & 61,661 & 26,358
6 & ALL & 9 & Relapse no. 2, two prior regimens & 100 & 7,978 & 33 & 29
7 & ALL & 16 & Relapse no. 1, refractory, four prior regimens & + & 100 & 5,978 & 27 & 4
8 & ALL & 3 & Relapse no. 1, refractory, four prior regimens & 100 & 4,546 & 67,815 & 187,000
9 & ALL & 17 & Relapse no. 4, six prior regimens, allo-SCT × 2 & 100 & 1,448 & 876 & 401
10 & LBL & 14 & Relapse no. 1, refractory, three prior regimens & 100 & 3,856 & 4 & 3
11 & ALL & 5 & Relapse no. 2, refractory, six prior regimens, allo-SCT & + & 100 & 3,053 & 5,568 & 37,474
12 & ALL & 16 & Relapse no. 1, refractory, four prior regimens & 100 & 4,371 & 46,540 & 26,640
13 & BL & 17 & Refractory, four prior regimens, auto-SCT & 100 & ND & 0 & 0
14 & ALL & 4 & Relapse no. 2, refractory, five prior regimens & 100 & 3,402 & 1 & 588
15 & ALL & 11 & Relapse no. 2, refractory, seven prior regimens & + & 75-82 & 5,640 & 34 & 2,385
16 & ALL & 18 & Relapse no. 2, refractory, five prior regimens & + & 100 & 9,380 & 12,880 & 33
17 & ALL & 19 & Relapse no. 2, refractory, four prior regimens, allo-SCT & + & 100 & 7,070 & 276 & 2
18 & ALL & 4 & Relapse no. 1, refractory, five prior regimens & + & 100 & 6,577 & 2 & 0
19 & ALL & 11 & Relapse no. 2, refractory, six prior regimens & + & 100 & 4,721 & 164 & 136
20 & ALL & 9 & Relapse no. 2, refractory, four prior regimens & 100 & 7,549 & 6 & 2
21 & ALL & 8 & Relapse no. 2, refractory, four prior regimens & 100 & 3,849 & 988 & 268
22 & ALL & 10 & Relapse no. 2, refractory, three prior regimens & 100 & 731 & 22,735 & 200
23 & ALL & 18 & Relapse no. 2, four prior regimens, allo-SCT & 100 & 7,585 & 1,398 & 10

\hline
\end{tabular}
\caption{Subject characteristics}
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Abbreviations: ANC, absolute neutrophil count; ID, identification number; Dx, diagnosis; PB: peripheral blood; BM, bone marrow; Nab, neutralizing antibody development; pre, pretreatment; post, posttreatment; allo-SCT, allogeneic stem cell transplant; auto-SCT, autologous stem cell transplant; QOD, every other day; Q, every; PD, progressive disease; BL, Burkitt lymphoma; ND, not done; CNS, central nervous system; LBL, lymphoblastic lymphoma; PET, positron emission tomography; LDH, lactate dehydrogenase; hypo, hypocellular.

\textsuperscript{*}Progressive extramedullary disease (renal, intraorbital masses) determined retrospectively after cycle no. 2.

\textsuperscript{6} http://ctep.cancer.gov/reporting/ctc.html
lasting >5 d or any platelet transfusion. Subjects with abnormal blood counts due to bone marrow infiltration were not evaluable for hematologic toxicity.

Pharmacokinetics. Plasma levels of BL22 were determined by incubating dilutions of plasma with Raji cells and comparing cytotoxicity as assessed by [3H]leucine incorporation to that obtained by a BL22 standard as previously described (15). Samples were obtained before, immediately after, and at 1/2, 1, 1 1/2, 2, 4, 8, 12, and 18 h after each day 1 dose, and before and immediately after subsequent doses. Area under the curve was calculated on the first dose of the cycle from either a monoexponential or biexponential model based on Aikake’s rule (20). For biexponential kinetics, the β half-life was used in analyses.

Neutralizing antibody assay. To assay for the presence of neutralizing antibodies, mixtures containing 90% serum and 10% BL22 (final BL22 concentration, 1,000 ng/mL) were incubated at 37°C for 15 min, diluted, and cultured with Raji cells, and the percentage of inhibition in the presence of subject serum was calculated as previously described (15). High-titer neutralizing antibodies were defined as levels that resulted in >75% neutralization of 1,000 ng/mL of BL22 in this assay.

<table>
<thead>
<tr>
<th>Table 1. Subject characteristics (Cont’d)</th>
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<tr>
<td><strong>BM blasts</strong></td>
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<tr>
<td>Pre</td>
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<td>&gt;90%</td>
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<td>&lt;5%</td>
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<td>&gt;90%</td>
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<tr>
<td>72%</td>
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<td>&gt;60%</td>
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<td>&gt;90%</td>
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<tr>
<td>79%</td>
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<td>30-70%</td>
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<td>78%</td>
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Response criteria. Standard disease-specific clinical and laboratory response criteria were used (21, 22).

Statistical analyses. For mice evaluated in preclinical studies, the probability of survival as a function of time, according to treatment administered, was determined by the Kaplan-Meier method with a log-rank test used to determine the difference of the probability of survival between the three pooled control groups and the combined BL22-treated groups. For the dose effect, pooled data from the two higher dose level groups were analyzed compared with the lowest dose. Results were not adjusted for multiple comparisons.

The relationship between clearance and peripheral blast count per microliter in subjects treated on the clinical trial was determined using Spearman rank correlation, with $|r| > 0.70$ indicating a strong correlation and the $P$ value indicating a result from a test of whether $r = 0$. Changes in peak BL22 levels between the first and last dose were evaluated using a paired $t$ test after verifying that the differences followed a normal probability distribution. Although patients received a varying number of doses, the difference is meant to illustrate the effect associated with maximal dosing on the trial. All $P$ values were two-tailed and are reported without adjustment for multiple comparisons.

Human subjects protections. All studies were approved by the Investigational Review Boards of the NCI (preclinical studies, clinical trial NCT00077493) or Emory University School of Medicine (preclinical studies).

Results

CD22 expression. To assess the frequency of CD22 expression in B-lineage ALL, blasts from 93 children with pre-B or Burkitt-type ALL were evaluated by flow cytometry. All cases were CD22+. CD22 expression distribution and density were further quantified in 54 of these cases. One hundred percent of the blasts within individual cases expressed CD22 in 52 of 54 cases. Minor populations of blasts without demonstrable CD22 expression were detected in two individuals (~80% and 90% CD22+). Determination of antibody binding capacity per cell revealed that the average CD22 site density within cases ranged from 451 to 16,523 sites per cell (median, 4,062), and only four cases showed <1,000 CD22 sites per cell (Fig. 1).

Cytotoxicity assays. In vitro cytotoxicity assays were conducted on blast samples obtained from 42 children with B-precursor ALL (Fig. 1). BL22-induced killing was
observed in all samples, with IC50s that ranged from 0.5 to 100 ng/mL (median, 9.8).

**Xenograft studies.** Murine xenografts treated with BL22 had a significant prolongation of leukemia-free survival (Fig. 1). Treatment cohorts were analyzed against the controls (using grouped data for both), and differences (treatment versus control) were significant ($P < 0.001$). To evaluate dose response, 3 and 4.5 μg cohorts were combined after determining that they had similar survival, and were analyzed compared with the 1.5 μg cohort. The difference was significant ($P < 0.05$).

**Phase I trial.** Twenty-three subjects ranging in age from 3 to 22 years (median, 13) were treated on the phase I trial. Twenty had ALL with marrow relapse, and one each had ALL with extramedullary relapse, stage 4 lymphoblastic lymphoma, and Burkitt lymphoma. All had been heavily pretreated, having received a median of four prior regimens (range, 2-9) and 20 were refractory to chemotherapy at the time of enrollment (Table 1). Twenty-two subjects completed at least one cycle of BL22 and one subject at dose level 7 received three of six doses. All subjects were evaluable through the DLT evaluation period for the primary study end points (i.e., toxicity, pharmacokinetics, and immunogenicity). During the course of the trial, the treatment schedule was amended to escalate the dose intensity (Table 1) based on safety and activity data from preceding cohorts. Cohort 5 was terminated early to increase the number of doses administered per cycle from three to six.

**Toxicity.** BL22 treatment was associated with an acceptable safety profile. No subject experienced infusion reactions, allergic events, vascular leak syndrome, or hemolytic uremic syndrome. All adverse events were self-limited and most were grades 1 and 2 (Fig. 2). Grades 3 and 4 events were rapidly reversible. Two subjects treated at dose level 7 experienced grade 4 ALT elevation of 1 to 2 days in duration, which met the original protocol definition of DLT. However, both of these resolved to levels required for ongoing treatment as scheduled supporting a revision in the DLT definition.
Pharmacokinetics. There was a dose-related increase in the peak plasma levels of BL22 with wide interpatient variation in pharmacokinetic parameters (Table 2). BL22 was rapidly cleared from the circulation with a plasma half-life on cycle 1 that ranged from 35 to 248 minutes (median, 106). Cycle 1 clearance correlated with increased peripheral blast count (Spearman correlation, \( r = 0.73; 95\% \) confidence interval, 0.45-0.88; \( P < 0.0001; \) Fig. 3). Peak plasma levels increased with progressive dosing in individuals who experienced blast reduction treated at the highest doses (30 and 40 \( \mu \)g/kg; \( P = 0.01; \) data not shown). This was in contrast to subjects without peripheral blast count reduction. Thus, pharmacokinetics seemed to be influenced by disease burden, consistent with rapid BL22 binding by CD22+ blasts.

Immunogenicity. Only 3 of 23 subjects (13%) developed neutralizing antibodies. One had preexisting low-titer antibodies that increased to 93% after cycle 1. Two developed \textit{de novo} antibodies with 78% neutralization after cycle 1 and 65% after cycle 3.

Clinical activity. Most subjects had high disease burden and rapidly progressive disease at the time of protocol enrollment. No responses were observed, however, transient clinical activity was seen in 16 of 23 subjects (70%), which in some cases was dramatic and clinically significant (Table 1; Fig. 4). For example, four subjects had >2 log\(_{10}\) reduction in circulating blasts and four had recovery of normal blood counts. Decreased blast infiltration of bone marrow (6) and extramedullary sites (3) were also observed. Notably, the most significant clinical activity was seen at the highest dose levels, including the four individuals with the largest reduction in peripheral blast counts (two each treated at dose levels 6 and 7) and all those with normalization of blood counts (treated at doses of 30 \( \mu \)g/kg or higher). Of the seven subjects without disease progression, two were ineligible to remain on the study (neutralizing antibodies, grade 4 ALT elevation), two chose to discontinue treatment after two to three cycles, and three developed progressive disease after two to three cycles, one of which was associated with the development of neutralizing antibodies.

Discussion

Despite significant progress in the curative treatment of childhood hematologic malignancies, relapse remains one of the greatest challenges in pediatric oncology (3). Furthermore, survivors have life-long risks of treatment-associated morbidity and mortality (6). New therapeutic approaches are needed to overcome chemotherapy resistance and to reduce side effects (23).

CD22 is rapidly internalized upon antibody or immunotoxin binding (24). As shown, this antigen is expressed in high frequency in childhood ALL. Unconjugated MoAbs may induce cytotoxicity by direct and indirect (e.g., immune-mediated) mechanisms, the latter of which are expected to be defective in individuals with ALL (25). Unconjugated MoAb against CD22 (epratuzumab) has recently been studied in childhood ALL and its activity as a single agent in the setting of relapsed ALL seems to be limited (26). Notably, anti-RFB4 control showed no activity against ALL xenografts or in cytotoxicity assays with primary samples from children with ALL (Fig. 1).

The activity of MoAbs can be dramatically increased by linkage to toxic moieties. Plant and bacterial toxins cause

### Table 2. Cycle 1 pharmacokinetic parameters

<table>
<thead>
<tr>
<th>Dose (( \mu )g/kg)</th>
<th>Subjects</th>
<th>Peak day 1 (ng/mL)</th>
<th>T(_{1/2}) (min)</th>
<th>AUC (( \mu )g * min/mL)</th>
<th>Vd (L/kg)</th>
<th>Clearance (mL/min/kg)</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>3</td>
<td>65 (61-107)</td>
<td>68 (40-146)</td>
<td>7 (4-22)</td>
<td>0.12 (0.10-0.14)</td>
<td>1.45 (0.45-2.11)</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>139 (64-341)</td>
<td>85 (35-120)</td>
<td>11 (7-36)</td>
<td>0.09 (0.02-0.14)</td>
<td>2.13 (0.55-2.98)</td>
</tr>
<tr>
<td>25</td>
<td>3</td>
<td>186 (112-413)</td>
<td>110 (101-136)</td>
<td>17 (12-66)</td>
<td>0.06 (0.02-0.08)</td>
<td>1.52 (0.38-2.14)</td>
</tr>
<tr>
<td>30</td>
<td>8</td>
<td>356 (85-500)</td>
<td>118 (43-248)</td>
<td>52 (5-116)</td>
<td>0.11 (0.03-0.28)</td>
<td>0.60 (0.27-343)</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
<td>469 (287-589)</td>
<td>108 (78-167)</td>
<td>53 (26-84)</td>
<td>0.10 (0.06-0.58)</td>
<td>0.81 (0.39-1.31)</td>
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NOTE. All values represent median (range).
Fig. 4. Hematologic improvement after BL22. A, improvement in the absolute neutrophil count (ANC) and platelet count during therapy. Arrows, BL22 treatment days. B, magnification, ×100 (Olympus BX51); C, oil magnification, ×1,000 (Olympus BX51). Bone marrow biopsies reveal a decrease in blast infiltration and an increase in normal hematopoietic precursors. Terminal deoxynucleotidyl transferase immunohistochemistry (blasts stain brown; subject no. 20).
cellular cytotoxicity through the inhibition of protein synthesis after internalization. These are highly potent and active in minute quantities, such that even a single molecule in the cytoplasm is sufficient to kill a cell (27). There have been limited studies of immunotoxins in childhood hematologic malignancies, and previously evaluated agents have been associated with severe adverse events and a high incidence of immunogenicity (28, 29). The clinical development of immunotoxins in general has been hampered by nonspecific toxicities, immunogenicity, and production complexities. Serial modifications in the Pseudomonas-based immunotoxin constructs used at the NCI have reduced nonspecific toxicities, increased stability, enhanced tissue penetration, and improved targeted cellular killing (30).

BL22 is a potent immunotoxin that targets CD22, which, as shown, is expressed in relatively high density on the surface of 100% of the blasts in the vast majority (96%) of cases of childhood B-lineage ALL. BL22 was shown to have clinical activity with acceptable toxicity in adults with relapsed and refractory hairy cell leukemia, in which a maximum tolerated dose of 40 μg/kg every other day for 3 doses every 28 days was defined (15). This pediatric phase I trial extends those observations and establishes that activity can be achieved in highly resistant childhood ALL with acceptable toxicity. Notably, BL22 was tolerated at a greater dose intensity (i.e., six doses every other day every 21 days) compared with adults, and hematolytic uremic syndrome, which was the DLT in adults, was not observed. Importantly, antileukemia activity was seen at all dose levels; however, clinical benefits in this highly refractory population were modest and transient at the doses tested. There are several possible explanations for the limited observed activity. Higher doses are likely required to achieve maximal benefit. Furthermore, although peak levels at the upper doses exceeded the concentrations required for in vitro cytotoxicity, drug exposure was limited in most subjects due to the rapid clearance associated with large disease burden. CD22 expression has been shown to be a determinant of response to BL22 in vitro (12), although there was no obvious influence of antigen density on clinical activity in this trial (Table 1). However, small numbers preclude definitive conclusions in this regard, and it is notable that all subjects without progressive disease had site densities that exceeded 3,000 sites per cell, whereas 6 of 16 with progressive disease had lower levels of expression.

This trial shows that BL22 can be administered at doses of up to 40 μg/kg every other day for 6 doses in children with ALL. No maximum tolerated dose was defined. Although two subjects treated at the highest dose level developed brief grade 4 ALT elevations, this was not dose-limiting given the short duration (1-2 days). We subsequently chose to close the trial and apply the schedule developed in this study (every other day for 6 doses every 21 days) to phase I testing of a modified BL22 construct with higher affinity for the CD22 antigen. This second-generation agent, HA22 or CAT-8015, was engineered to replace three amino acid residues in the heavy chain complementary determining region 3 of the BL22 binding domain. This modification increased the binding affinity for CD22 by 14-fold, which resulted in an approximately 1 log10 improvement in cytotoxicity against a variety of CD22+ malignancies (31, 32).

In summary, CD22 represents an excellent target for pediatric B-lineage hematologic malignancies. These studies offer proof-of-principle that anti-CD22 Pseudomonas-based immunotoxins can be administered to children and have the potential to overcome chemotherapy resistance and induce cytotoxicity of CD22+ blasts refractory to standard therapy. Anti-CD22 immunotoxins hold therapeutic promise in this common subtype of pediatric cancer.

Disclosure of Potential Conflicts of Interest

R.J. Kreitman, D.J. FitzGerald, and I. Pastan are coinventors on patents assigned to the NIH for the investigational product used in this research.

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