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Cytotoxicity of the anti-CD22 immunotoxin HA22 (CAT-8015) against paediatric acute lymphoblastic leukaemia

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Summary

Acute lymphoblastic leukaemia (ALL) remains the most frequent cause of cancer-related mortality in paediatrics and outcome is poor for patients who have high-risk ALL or relapse. HA22 (CAT-8015) is an immunotoxin composed of an anti-CD22 variable fragment linked to a 38 kDa truncated protein derived from *Pseudomonas* exotoxin A. Using a bone marrow mesenchymal cell culture assay to support ALL cell viability, we investigated the *in vitro* cytotoxicity of HA22 against ALL blasts from newly diagnosed (n = 13) and relapsed patients (n = 22). There was interpatient variability in sensitivity to HA22. Twenty-four of 35 patient samples tested were sensitive (median 50% lethal concentration 3 ng/ml, range 1–80 ng/ml). Blasts from the other 11 patients were not killed by 500 ng/ml HA22. The median 50% lethal concentration was 20 ng/ml for all patients. There was no significant difference in HA22 sensitivity between diagnosis and relapse samples but peripheral blood ALL blasts were more sensitive to HA22 than those from bone marrow (P = 0.008). Thus, HA22, at concentrations achievable in patients, is highly cytotoxic to B-lineage ALL cells. These results provide a strong rationale for clinical testing of

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Disclosure of potential conflict of interest

Ira Pastan is a co-inventor on patents assigned to the NIH for the investigational product used in this research.

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this agent in children with drugresistant ALL and offers the potential to reduce morbidities of treatment while improving outcome.

Keywords

acute lymphoblastic leukaemia; childhood cancer; CD22; immunotoxin; apoptosis

Acute lymphoblastic leukaemia (ALL) is the most common paediatric malignancy (Linaberry & Ross, 2008). Although significant progress has been made over the last 50 years, ALL remains the most frequent cause of cancer-related mortality in children (Pollack *et al*, 2007; Pui *et al*, 2009). Long-term disease-free survival rates are particularly poor for patients with certain leukaemia subtypes and for those who relapse (Gloeckler Ries, 1999; Gaynon, 2005). For these patients, new treatment approaches are urgently needed. For patients in lower risk groups, where cure is more likely, morbidities during and after treatment remain an important problem highlighting the need to replace toxic non-specific chemotherapy to improve the outcome for long-term survivors (Oeffinger *et al*, 2006).

Immunotoxins are novel therapeutic agents for haematological malignancies. They typically comprise a monoclonal antibody or a cell antigen-binding antibody fragment and a toxin moiety that induces cell death. Our laboratory has developed immunotoxins in which the variable fragment (Fv) of an antibody is linked to a 38 kDa truncated derivative (PE38) of *Pseudomonas* exotoxin A, obtained by removing its natural cell binding domain (Domain I) (Pastan *et al*, 2006).

To develop an immunotoxin that would be effective to treat B cell malignancies, we selected CD22, a member of the sialic-acid binding immunoglobulin-like lectin (Siglec) family of adhesion molecules (Nitschke, 2009) as a target. CD22 is expressed on virtually all malignant B cells, including pre-B and mature B ALL blasts, and the CD22 antigenimmunotoxin complex is rapidly internalized (Du et al, 2008). BL22 is a first generation anti-CD22 immunotoxin composed of the Fv portion of the anti-CD22 monoclonal antibody RFB4 fused to PE38 (Mansfield et al, 1996). Previous studies have shown that BL22 is cytotoxic against CD22-positive malignant cells in vitro and in vivo (Kreitman et al, 2000, 2005). HA22 (CAT-8015) is a second generation, higher affinity anti-CD22 immunotoxin made through targeted mutations in the hotspot region of the complimentarity determining region-3 (Salvatore et al, 2002). Upon binding to CD22, HA22 is internalized and after processing, a portion of the toxin is transferred to the endoplasmic reticulum and translocated into the cytosol. Here the toxin catalyses the ADP-ribosylation and inactivation of elongation factor-2, resulting in inhibition of protein synthesis and cell death. In this study, we determined the cytotoxicity of HA22 against paediatric ALL using a novel bone marrow stromal assay system.

Materials and methods

ALL samples

Blood and bone marrow samples were obtained from 35 patients with B-lineage ALL (Table SI) treated at the National Cancer Institute (NCI), St Jude Children's Research Hospital

(SJCRH) or Johns Hopkins Hospital (JHH) with informed consent. The majority (n = 22) were obtained from individuals with multiply relapsed ALL who were referred to the NCI for Phase I clinical trial participation. Thirteen patient samples from initial diagnosis were randomly selected from those available in the tumour banks at SJCRH and JHH. Thirty-three cases were characterized as pre-B ALL by flow cytometry and two as Burkitt-type/mature B-cell ALL. In all cases, >80% blasts expressed CD19 and CD22 antigens by flow cytometry. Cells were cryopreserved in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 10% dimethyl sulfoxide. Institutional review board approval was obtained for the use of these samples in these studies.

Culture conditions

To measure the cytotoxic activity of the anti-CD22 immunotoxins HA22 and BL22, we cultured cells on bone marrow-derived mesenchymal cells as previously described (Campana *et al*, 1993). Briefly, human bone marrow stromal cells that had been immortalized by telomerase transfection (developed at St Jude Children's Research Hospital) were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal calf serum (Sigma-Aldrich, St Louis, MO, USA). 2×10^4 Stromal cells were plated into each well of flat-bottomed 96-well plates and cultured until confluent. On day 1 of the assay, ALL cells were resuspended in RPMI-1640 medium, 10% heat-inactivated FBS, glutamine (1·) and sodium pyruvate (1×). 3×10^5 ALL cells were added to each well of stroma-coated plates. On day 2, BL22 or HA22 were added at final concentrations of 0, 0·5, 1, 5, 10, 50, 100 and 500 ng/ml in duplicate wells. SS1P (50 ng/ml), an anti-mesothelin *Pseudomonas* immunotoxin, was used as a negative control. The cytotoxicity of dexamethasone (10 µmol/l) was also tested in this assay. Cells were incubated for a further 72 h in a humidified atmosphere at 37°C with 5% CO₂.

Flow cytometric analysis

The cells were harvested by vigorous pipetting, transferred to Falcon tubes (Cat. No. 352052; BD Biosciences, San Jose, CA, USA) and labelled with mouse anti-human CD19 antibody conjugated to fluorescein isothiocyanate (Cat. No. 555412; BD Pharmingen, San Jose, CA, USA). The cells were resuspended in 1× Annexin Binding Buffer and labelled with 7-Aminoactinomycin D (7-AAD) and Annexin conjugated to phycoerythrin (PE) (Cat. No. 559763; PE Annexin V Apoptosis Detection kit I, BD Pharmingen). The cells were analysed with a FACScalibur flow cytometer in combination with CellQuest (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (Tree Star Inc., Ashland, OR, USA).

Viability was assessed by setting gates based on the light scatter properties of the blasts (Figure S1). Due to spontaneous cell death, the relative percentage of viable cells at the end of the assay was calculated using the following formula: (no. of CD19⁺ viable cells recovered in test well/no. of CD19⁺ viable cells in untreated well \times 100). All results represent the mean of duplicate experiments.

The 50% lethal concentration (LC_{50}) was defined as the concentration of HA22 that killed 50% of the viable cells at the termination of the assay.

Cell death by apoptosis was studied for all samples and late stage apoptosis was defined as cells positive for 7-AAD and Annexin-PE by flow cytometry. Non-apoptotic cells were those negative for 7-AAD and Annexin-PE.

Antigen site density was quantified by determining the anti-CD22 antibody binding capacity per cell (Schwartz *et al*, 1998). ALL samples were stained under saturating conditions with anti-CD22 antibody with 1:1 antibody to PE conjugation (BD Biosciences) using the BD Biosciences QuantiBRITE system for fluorescence quantitation. The antibody binding capacity value is the measurement of the mean value of the maximum capacity of each cell to bind the anti-CD22 antibody. QuantiBRITE beads are pre-calibrated standard beads containing known levels of PE molecules. QuantiBRITE beads were acquired on a FACSCalibur flow cytometer on the same day at the same instrument settings as the individual specimens. A standard curve comparing the geometric mean of fluorescence to known phycoerythrin content of the QuantiBRITE beads was constructed using QuantiCALC software.

Immunotoxins and chemotherapy agents

The recombinant immunotoxins HA22, BL22 and SS1P were produced as previously described (Pastan *et al*, 2004). Dexamethasone was provided by the Division of Veterinary Resources (NIH).

Statistical analysis

An exact Wilcoxon rank sum test was used to determine the statistical significance of the difference in unpaired observations between two groups of subjects. For comparison of LC_{50} values between relapsed and newly diagnosed patients, an exact log-rank test was used because many of the observations were censored at HA22 500 ng/ml concentration. A Wilcoxon signed rank test was used to determine whether the LC_{50} ratios of BL22/HA22 were equal to 1·0. Correlations between parameters were evaluated using Spearman rank correlation analysis. All *P*-values are two-tailed and reported without adjustment for multiple comparisons. *P*-values <0·05 were considered to represent statistically significant effects.

Results

Cytotoxicity of HA22 against paediatric ALL cells

The cytotoxicity data of HA22 against 35 cryopreserved patient samples is summarized in Fig 1 (Data in Table SII and representative killing curves are shown in Figure S2). Samples varied in their sensitivity, ranging from 100% cell death to almost complete resistance to killing at 500 ng/ml HA22. Greater than 75% killing was achieved in 18 of 33 patient samples tested. There was no significant difference in LC_{50} or the percentage of viable cells after HA22 when compared to blasts from relapsed and newly diagnosed patients (Fig 1, P = 0.69 and P = 0.80, respectively). Of the 22 relapse samples, eight were very sensitive to HA22 with LC_{50} 5 ng/ml, seven had a moderate response with LC_{50} s ranging from 18 to 80 ng/ml and seven samples were more resistant to HA22 with 50% killing not achieved. Cells from 13 newly diagnosed patients also showed a range of sensitivity to HA22. LC₅₀s

ranged from 0.3 to 60 ng/ml (median 3 ng/ml) in nine samples, with seven showing extreme sensitivity (LC₅₀s 3 ng/ml). Four samples appeared more resistant to HA22 and 50% killing was not achieved at the 500 ng/ml concentration. The median LC₅₀ was 20 ng/ml for all patient samples tested.

Relation between sensitivity to HA22 and clinical and cellular features

There was no apparent association between HA22 cytotoxicity and the age, sex, diagnostic white cell count or patient outcome following standard chemotherapy. We studied the relationship between response to HA22 and number of CD22 sites per cell. CD22 site density, measured in 19 samples, ranged from 451 to 15 217 (median 4063), and was only weakly correlated with HA22-induced cytotoxicity (r = 0.33, P = 0.16) (Fig 2).

To assess whether the anatomical origin of ALL blasts might affect response to HA22, samples derived from peripheral blood (n = 9) and bone marrow (n = 26) were compared for cytotoxicity (Fig 3). In general, peripheral blood ALL blasts were more sensitive to HA22 than bone marrow blasts (3.6-fold difference in the percentage of viable cells, P = 0.008).

Specificity of HA22 cytotoxicity and mechanism of action

To ensure that the cytotoxicity observed was due to specific binding of HA22 to CD22, an anti-mesothelin *Pseudomonas* immunotoxin SS1P was used as a negative control. SS1P at 500 ng/ml showed no cytotoxicity.

We also compared the activity of HA22 to the lower affinity reagent BL22 in a subset of samples found to be sensitive to HA22. As expected, HA22 was more active than BL22 in all samples tested (Table I).

ALL cells were analysed for externalization of membrane phosphatidylserine and 7-AAD binding (Figure S3) to measure apoptosis. In all cases HA22 caused a dose-dependent increase in the percentage of cells undergoing apoptosis. There was close correlation of the percentage of viable ALL cells after treatment between light scatter properties and annexin/7-AAD staining for all samples.

Cytotoxicity of HA22 compared to dexamethasone

Dexamethasone, at a concentration of 10 µmol/l, had previously been shown to induce apoptosis in the majority of ALL patient samples cultured on stromal cells (Ito *et al*, 1996). Different patterns of cytotoxicity were seen and patient samples showed a range of sensitivities to dexamethasone (Table SII; examples are shown in Figure S2). The percentage of viable ALL cells at 10 µmol/l dexamethasone ranged from 4% to 158%, with a median of 40% (median of 29% for ALL samples from diagnosis and 42% for relapse samples, P= 0·2). HA22 was equally or more cytotoxic than dexamethasone in 17 patients. Resistance to HA22 did not correlate with dexamethasone resistance (r= 0·29, P= 0·09). HA22 could be cytotoxic to both dexamethasone-resistant and dexamethasone-sensitive ALL blasts in a dose-dependent manner. Resistance to dexamethasone correlated with resistance to HA22 for newly diagnosed patients. There was no significant difference in sensitivity of blasts from blood or bone marrow to dexamethasone (P= 0·27). To confirm that HA22 and dexamethasone were not cytotoxic to the stromal cells, confluent stromal cells were incubated with these agents for 72 h (500 ng/ml and 10 μ mol/l, respectively). At the end of the assay the stromal cells were incubated with WST-1. There was no difference in stromal cells incubated with HA22 or dexamethasone compared to untreated controls (data not shown). The stromal layers also remained intact without obvious morphological changes when microscopically examined.

Discussion

The aim of this study was to assess whether HA22 was cytotoxic against B-lineage ALL blasts from paediatric patients. We hypothesized that HA22 would kill paediatric pre-B ALL blasts in a dose-dependent manner at concentrations achievable in patients. The study is the first to report *in vitro* evidence that this novel anti-CD22 *Pseudomonas* immunotoxin is cytotoxic to blasts from children with B-precursor ALL, the most common paediatric cancer subtype. This work also established the mechanism of the observed cytotoxicity. The majority of blasts were sensitive to HA22 and cell death occurred via apoptosis. Furthermore, HA22 was cytotoxic to relapsed, newly diagnosed and dexamethasone-resistant patient samples.

Although anti-CD22 immunotoxins have been shown to have activity in adults with hairy cell leukaemia, efficacy against paediatric ALL has not been demonstrated (Kreitman *et al*, 2000). LC₅₀ values ranged from 1 to 80 ng/ml, which are well below the plasma level achieved in paediatric patients with ALL treated at the upper dose levels on Phase I trials of BL22 (85–500 ng/ml) (Wayne *et al*, 2010) and HA22 (range 311–586 ng/ml) (Wayne *et al*, 2009). Additionally, HA22 was cytotoxic to blasts that were expected to be chemotherapy-resistant, such as those from relapsed patients and those resistant to dexamethasone. These data suggest that the mechanisms of resistance to standard chemotherapy are different than those for PE38-induced death.

HA22-induced cytotoxicity of ALL blasts occurred by apoptosis. PE38-mediated cell death has been shown to occur by induction of apoptosis and by non-caspase mediated mechanisms (Keppler-Hafkemeyer *et al*, 2000; Kreitman et al, 2000). HA22 had greater activity than BL22: one case showed a 10-fold decrease in the LC₅₀. Increasing the affinity of the immunotoxin for CD22 appears to increase the cytotoxicity and results are consistent with those observed in CLL (Salvatore *et al*, 2002).

Resistance to immunotoxins could be affected by variation in the rates of internalization between patient samples. Du *et al* (2008) found that, for different lymphoma cell lines, the majority of BL22 was internalized within 15 min. However, in those studies the number of CD22 sites per cell ranged from 26 000 to 94 000 (Du *et al*, 2008). The number of CD22 sites per cell in our studies ranged from 451 to 15 217 and site density was only weakly correlated with HA22-induced cytotoxicity. Thus, the number of CD22 sites available for immunotoxin binding may be a factor in ALL blast sensitivity to HA22. After internalization, the PE38 toxin must be cleaved from the Fv fragment and transported into the cytosol. It is possible that the efficiency of intracellular processing and the localization of immunotoxin to endocytic or lysosomal compartments affect the response to HA22. The

importance of lysosomal degradation in ALL and the variation between patient samples is unknown.

Cytotoxicity was compared in peripheral blood- and bone marrow-derived blasts. HA22 appeared to be more cytotoxic to peripheral blood blasts, raising important biological questions. The result should be interpreted cautiously as samples were not paired and the numbers assayed were small. Cell surface receptors, such as CXCR4, are involved in homing and maintaining blasts in the haematopoietic niche and altered expression may contribute to blast localization to the perivascular space and migration into the peripheral blood. Peripheral blood blasts may lose stromal-cell interactions and/ or dependency, which in turn could lead to changes in responsiveness to therapy.

The finding that HA22 is cytotoxic to blasts from children with ALL may have important clinical implications. Acute and late morbidities associated with standard treatment of ALL remain significant and those individuals who relapse have a particularly poor prognosis. New therapies that can overcome resistance to standard chemotherapy agents without adding significant toxicities are needed. This study showed that HA22 is cytotoxic to blasts from most patients and non-specific toxicities are expected to be less in comparison to chemotherapy. Serial modifications in the *Pseudomonas*-based immunotoxin constructs utilized at the NCI have reduced such non-specific toxicities (Pastan, 2003). Importantly, CD22 is only expressed on cells of B-lineage, further reducing the risk of non-specific side effects. The toxicity profile of anti-CD22 Pseudomonas immunotoxins has been encouraging to date (Kreitman et al, 2001, 2005; Wayne et al, 2009, 2010). The most frequent adverse events have been reversible increases in hepatic transaminases and hypoalbuminaemia. In Phase I testing in paediatric subjects, BL22 was associated with an acceptable safety profile. All adverse events were self-limited, most were Grade I and 2, and no dose-limiting toxicities were observed (Wayne et al, 2010). HA22 appears to have a similar toxicity profile to BL22 (Wayne et al, 2009).

Studies of other CD22-targeted therapies in paediatric ALL have recently been reported. Epratuzumab, a humanized monoclonal antibody against CD22, is currently undergoing study in children with relapsed ALL by the Children's Oncology Group (Raetz *et al*, 2008). Its activity as a single agent in that setting appears to be limited. A Phase I study of a mixture of ricin-based immunotoxins against CD22 and CD19 (Combotox) was recently conducted for paediatric patients with ALL. In that trial, clinical activity was observed, although this agent was associated with severe adverse events and a high incidence of immunogenicity (Herrera *et al*, 2009). Other anti-CD22-targeted agents are in development. For example, a calicheamicin immunoconjugate, CMC-544 (Inotuzumab ozogamicin), has been shown to be active in pre-clinical models of ALL and in clinical trials in adults with non-Hodgkin lymphoma (DiJoseph *et al*, 2008).

In vitro leukaemia-stromal cell assays have been shown to be predictive of treatment outcome (Kumagai *et al*, 1996; Galderisi *et al*, 2009), which offers the possibility of utilizing such in the development of individualized leukaemia therapies in the future. This study also established the bone marrow stromal cell assay system as a method that can be utilized to

further study the biological basis for sensitivity and resistance of ALL blasts to targeted immunotoxins.

In conclusion, the anti-CD22 immunotoxin HA22 has *in vitro* activity against blasts from children with newly diagnosed and relapsed ALL. These data provide experimental support for ongoing clinical trials of this agent for paediatric ALL (ClinicalTrials.gov number: NCT00659425; http://clinical-trials.gov/ct2/show/NCT00659425) with the potential for a new active agent with reduced non-specific toxicity. Future studies will include investigating the mechanisms of resistance in paediatric ALL and whether this can be overcome with combination therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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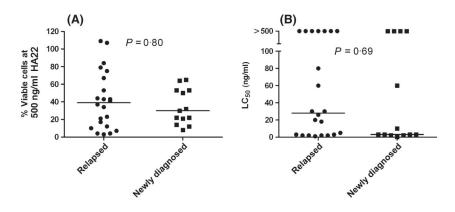
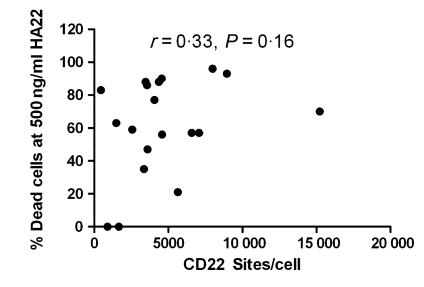
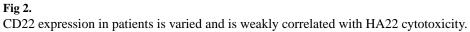
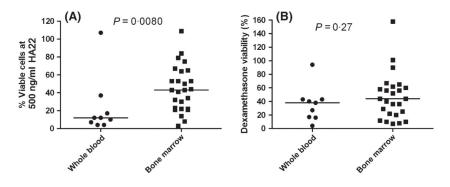


Fig 1.

No difference in cytotoxicity of HA22 against paediatric ALL cells from relapsed and newly diagnosed patients. (A) Scatter plot of the percentage of viable ALL cells remaining after 72 h incubation with 500 ng/ml HA22 for samples. (B) Scatter plot of the 50% lethal concentration (LC_{50}) results for HA22.









Cytotoxicity of HA22, but not dexamethasone, appears to be greater against blasts from peripheral blood compared to those from bone marrow.

Table I.

Cytotoxicity of BL22 and HA22 against patient samples.

Patient	LC ₅₀ (ng/ml)		LC ₅₀ ratio (ng/ml)
	BL22	HA22	BL22/HA22
5	20	3	6.7
9	3	2	1.5
10	3	2	1.5
20	20	5	4.0
22	Not achieved	60	>8.3
29	1	0.3	3.4

LC50, 50% lethal concentration.

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