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Wide variability in the time required for immunotoxins to kill B lineage acute lymphoblastic leukemia cells: Implications for trial design

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Abstract

Purpose—Recombinant immunotoxins (rITs) targeting CD22 are highly active in hairy cell leukemia, but less so in acute lymphoblastic leukemia (ALL). This study aims to understand the variable activity of a rIT against ALL towards improving responses in clinical application.

Experimental Design—We determined *in vitro* activity of rITs by WST-8 assays and the time needed to kill ALL cell lines and patient-derived ALL blasts by flow cytometry. The findings were translated into two systemic ALL xenograft models. Differences in time needed to kill KOPN-8 cells for distinct rITs were addressed biochemically.

Results—*In vitro* activity (IC₅₀) of anti-CD22 rIT varied 210-fold from 0.02 to 4.6 ng/ml. Activity also varied greatly depending on the time ALL cells were exposed to immunotoxin from <30 min to >4 days. For KOPN-8, the difference in exposure time was related to intracellular rIT-processing. We showed in newly developed ALL xenograft models, where immunotoxins have a short half-life, that the needed exposure time *in vitro* predicted the responses *in vivo*. By replacing bolus dose with small doses at frequent intervals or with continuous infusion, responses were substantially improved. We confirmed exposure time variability on patient-derived ALL samples and showed a correlation between exposure time needed to reach maximal cytotoxicity *in vitro* and their clinical response.

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Conflict-of-Interest

A.S.W. and I.P. are co-inventors on patents assigned to the NIH for the investigational products. A.S.W. has received from Medimmune: research support, honorarium, travel support; Pfizer, Kite Pharma, and Spectrum Pharmaceuticals: honorarium and travel support. F.M., T.C., X.F.L. and I.P. declare no financial interests.

Conclusion—The exposure time needed for rITs targeting CD22 to kill ALL cells varies widely. Our results suggest that ALL patients would have a better response rate to anti-CD22 immunotoxins if treated by continuous infusion rather than by bolus injections.

Keywords

Immunotoxin; acute lymphoblastic leukemia; targeted therapy; CD22; Moxetumomab pasudotox

Introduction

Current therapy for childhood acute lymphoblastic leukemia (ALL) results in 80% long-term remissions (1). The treatment of relapse remains challenging and is based on intensified treatment often followed by bone marrow (BM) transplantation (2). Relapse after second line is even more difficult to treat and cure is rare (3, 4). Because multiply relapsed ALL is often broadly chemotherapy resistant (5), promising new treatment options based on CD19-targeting (e.g. CAR-T cells (6-8), CD19-BiTE (9-11)) or CD22-targeting (12-15) are being developed. Optimization of the antibody-based therapies currently in clinical development might further improve the already promising results.

We have generated recombinant immunotoxins (rITs) composed of an anti-CD22 antibody fragment fused to a truncated *Pseudomonas* exotoxin A (PE) (16). CD22 is expressed on many B-cell malignancies including B-lineage ALL (17), Burkitt lymphoma (BL), hairy cell leukemia (HCL), and mantle cell lymphoma (18). The first CD22-targeting rIT, BL22 (CAT-3888), showed major clinical responses in HCL, but was less active in ALL (19, 20). HA22 has a 10-fold higher affinity for CD22 than BL22 resulting in higher activity *in vitro* and *in vivo* (21). HA22, also known as CAT-8015 or Moxetumomab pasudotox (22) is active against HCL with response rates of 85% (20). In a pediatric phase I clinical trial, HA22 showed an objective response in 15 of 46 (33%) children with ALL (23). Although this single-agent response rate of 33% in individuals with multiply relapsed ALL is noteworthy, we had expected more responses because CD22 is uniformly expressed on the surface of B-lineage ALL (17) and HA22 is cytotoxic *in vitro* against blasts from the majority of patients with relapsed and chemotherapy-refractory ALL (24).

In attempt to improve CD22-targeting rITs further, we constructed the new immunotoxin LMB-11. It has an anti-CD22 Fab, a deletion of most of PE-domain II except for the furin processing site, and seven mutated amino acid residues in domain III (Fig. 1A & B) (25). The mutations were introduced to disrupt immunogenic epitopes and strongly diminished rIT binding by patient-derived neutralizing antibodies (25). The removal of most of domain II allows much higher dosing in animals without inducing liver damage or capillary leak syndrome (26). LMB-11 has been tested in mice bearing subcutaneous BL (CA-46) where it produced sustained complete remissions; while HA22 at its maximum tolerated dose did not (25). These results prompted us to test LMB-11 on ALL cell lines *in vitro* and in systemic ALL xenograft models, which we then compared to the activity of HA22 with the aim of improving responses.

Materials and Methods

Cell lines

The cell lines CA46 (27), KOPN-8, SEM, REH, Nalm-6 (12), and HAL-01 (28) were described previously. These cell lines have been tested and authenticated by str analysis. All cell lines were grown in RPMI-1640 with 10% FBS, 100 U penicillin, and 100 mg streptomycin (Invitrogen, Carlsbad, CA).

Reagents

HA22 (21) and LMB-11 (25) were produced as described.

rITs were labeled with Alexa Fluor 647 Labeling Kit (Invitrogen) according to manufacturer's instructions. rITs for *in vivo* assays were diluted in phosphate buffered saline (PBS).

Secondary antibodies were purchased from Santa Cruz (Dallas, TX), primary antibodies (MCL1, PARP, EF2, GAPDH) from Cell Signaling (Danvers, MA), flow cytometry antibodies and Annexin V-PE/7-AAD from Becton Dickinson (Franklin Lakes, NJ).

Cell assays

Cell growth arrest was measured by WST-8 as described (25). 5,000 cells/well were incubated with various rIT concentrations for 72 hours. WST-8 reagent was added and assays analyzed after 2 hours. Values were normalized between Cycloheximide (10 µg/ml final, Sigma-Aldrich, St. Louis, MO) and RPMI. Non-linear regression was done using GraphPad Prism to obtain IC₅₀ concentrations.

LMB-11-Alexa647 uptake was performed with 1 million cells/ml using indicated concentrations of rIT-Alexa647 at 37°C. Surface bound molecules were stripped for 10 minutes in 0.2 M Glycine pH 2.5, cells washed twice with PBS and analyzed by flow (FACS Calibur, Becton Dickinson). Internalized rIT-molecules were quantified with Alexa647-Quantum beads (Bangs Laboratories, Fishers, IN) as described (29). Beads were used to create a standard curve for conversion of MFI into absolute molecule number. MFI of test samples was interpolated to define absolute number of rIT-Alexa647 molecules.

For *in vitro* apoptosis assays, 1 million cells/ml were incubated with 2.8 nM rIT for various times, cells washed twice, and transferred to a new plate. Seventy-two hours after assay initiation (seven days for CA46 cells), cells were washed, stained with 7-AAD/Annexin-PE, and analyzed by flow. Results were analyzed with FlowJo software (Tree Star, San Carlos, CA).

For primary patient cell assays, we plated 20,000 OP-9 stromal cells per well in a 24-well plate in α-MEM (1% P/S, 20% FBS) on Day 0. On Day 1, 300,000 patient cells were added and 2.8 nM rIT 4 hours later. At indicated times, cells and wells were washed and re-plated in the same co-culture wells. Seventy-two hours after initiation, cells were stained with anti-hu-CD19 and Annexin V-PE/7-AAD and analyzed by flow.

Inhibition of protein synthesis was determined by [³H]-leucine incorporation as described (30). One million cells/ml were plated in complete RPMI and treated for indicated times. Cells were pulsed with 2 µCi [³H]-leucine for 1 hour, frozen on dry ice for 30 minutes, thawed at 37°C and read using a liquid scintillation counter.

Cell-free ADP-ribosylation was performed as described (31). One million cells were incubated with 2.8 nM rIT for various times. Cells were washed and lysed in 100 µl of modified RIPA buffer (50 mM TrisHCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, HALT inhibitors). 12.5 µg protein was incubated with 100 ng of LMB-11 and biotinylated NAD (Trevigen, Gaithersburg, MD) for 1 hour at room temperature. Protein was separated under reducing conditions by gel electrophoresis, blotted, and NAD-Biotin was detected by 1:200,000 Streptavidin-HRP (Jackson-Immuno, West Grove, PA). The cell lysates were also analyzed by western blot.

Intracellular furin-cleavage was analyzed as described (32). One million KOPN-8 cells were treated with 2.8 nM rIT, lysed at various times, and lysates were separated under reducing conditions by gel electrophoresis, followed by western blot. rIT was probed for with in-house produced polyclonal rabbit anti-PE38.

Animal studies

Animals were handled according to NIH guidelines; studies were approved by the NCI Animal Care and Use Committee.

Five million KOPN-8 or HAL-01 cells were injected on Day 1 via tail vein into 6- to 8-week-old NSG mice (NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ*). Immunotoxin was given IV or via osmotic pumps IP at doses and schedules indicated. To assess early response, mice were euthanized 3 days after the last dose. For survival studies mice were followed until disease progression (weight loss >10%). Seven day ALZET pumps were loaded with 1 mg/ml HA22/PBS under sterile conditions. Pumps were implanted surgically into the peritoneal cavity according to manufacturer's instructions. Mice were euthanized 7 days after implantation.

BM was extracted by flushing femurs. Human ALL was stained with anti-human-CD19-FITC. Experiments on mouse-derived tissue were F_c-receptor blocked with anti-murine CD16/32. For *in vivo* biodistribution of LMB-11-Alexa647, a single dose of 2.5 mg/kg was injected on day 14, mice sacrificed at indicated times, and fluorescence Alexa647 in the CD19-positive population determined by flow.

Patient samples

Primary ALL blast samples from eight patients with CD22-positive pre-B-ALL treated on a phase I trial of HA22 (NCT01891981) were collected before the first HA22 dose under protocols approved by the NCI Institutional Review Board. In liquid nitrogen stored samples were thawed, washed, resuspended in PBS and injected IV in NSG mice. Mice were euthanized at disease progression, and whole spleen and BM (spine, hip, and femur) extracted. Cells were analyzed for hu-CD19, hu-CD22, and hu-CD10, viably frozen in RPMI-1640 containing 10% DMSO and stored in liquid nitrogen.

Statistics

Results were analyzed in GraphPad Prism v6.01. Two group comparisons were done using unpaired t-tests and multiple comparison analyses by ANOVA as indicated.

Results

KOPN-8 mouse model shows lower than expected response to LMB-11

To test if LMB-11 was as active in ALL as in the CA46 model (25), we established a mouse human ALL xenograft model. We screened cell lines for their sensitivity to LMB-11 *in vitro* (Table 1). Based on the initial hypothesis that cells with similar IC₅₀s should respond alike in an animal model, we selected KOPN-8 because it had an IC₅₀ comparable to CA46 (0.8 vs. 0.4 ng/ml) and because it grew rapidly in murine BM after IV injection. We treated KOPN-8 bearing mice with either vehicle or LMB-11 at 2.5 mg/kg every other day (QOD) for five injections, almost twice the dose given in the CA46 model (25), and found that LMB-11 only produced stable disease (SD) during treatment (Fig. 1C). In vehicle treated mice, KOPN-8 BM infiltration was 5% on day 8 and increased rapidly within 1 week to reach 85% infiltration. If treated with five doses of 2.5 mg/kg QOD starting from day 8, KOPN-8 BM infiltration remained approximately 5% during treatment and began increasing 4 days after the last dose.

Because we hypothesized that CA46 and KOPN-8 should respond similarly to treatment *in vivo*, achieving only SD was unexpected. To determine if the KOPN-8 cells became resistant in mice, we removed cells from the marrow and tested their sensitivity *in vitro*; the cells expressed CD22 and were still LMB-11 sensitive (Supplementary Fig. 1A & B). To be certain LMB-11 was reaching the cells, we determined the amount of rIT-molecules internalized into KOPN-8 cells in the BM after injecting 2.5 mg/kg LMB-11-Alexa647. The signal reached a maximal intensity after 1 hour and remained stable for up to 24 hours (Supplementary Fig. 2). Using Alexa647-beads, we determined that 20,500 LMB-11 molecules were internalized by KOPN-8 cells in the BM of treated mice. To correlate the number of molecules reaching KOPN-8 *in vivo* with the number of molecules needed to kill KOPN-8 *in vitro*, we generated a standard curve for 1 hour treated cells in cell culture and found a concentration dependent uptake of Alexa647-molecules (Fig. 1D). At KOPN-8's IC₅₀ of 0.8 ng/ml, only 220 LMB-11 molecules were internalized after 1 hour. The numbers of molecules taken up by KOPN-8 cells in the BM 1 hour after treatment was equivalent to a cell culture concentration of approximately 200 ng/ml of LMB-11. Thus, the number of toxin molecules that reached KOPN-8 in the BM was 93-fold higher than the number taken up within 1 hour at IC₅₀ concentrations *in vitro*. These data eliminate inadequate delivery of LMB-11 to ALL cells as a cause for the unexpectedly low response.

Exposure time is relevant *in vitro*

To determine if the short half-life of LMB-11 in mice (28 min) (25) is an explanation for the poor response, we determined *in vitro* how long cells need to be exposed to rITs for them to die. In contrast to our standard assay with continuous 72 hours exposure, cells were exposed to 200 ng/ml LMB-11 or equimolar 173 ng/ml HA22 for various times, washed, re-plated, and analyzed 72 hours after initiation of the experiment for cell death by flow cytometry. We

chose the 3-day time point because this interval provided enough time for cells to become Annexin V/7-AAD positive if immunotoxin had initiated cell death. We found that the rIT exposure time needed to induce >90% apoptosis in LMB-11 treated KOPN-8 was more than 9 hours (Fig. 2). Surprised by the long time period, we tested HA22 and found it completely killed KOPN-8 cells after less than 30 minutes exposure. We then screened a panel of cell lines, including four additional ALL (REH, HAL-01, SEM, Nalm-6) and one BL (CA-46).

Before determining the time these cells had to be exposed to rIT to induce apoptosis, we measured activity by WST-8 assays under 3-day continuous exposure (Table 1). As expected from earlier studies (25), rIT-activity varied. The activity of LMB-11 varied 23-fold (0.2 to 4.6 ng/ml), and that of HA22 169-fold (0.02 to 3.2 ng/ml). The activity of LMB-11 was generally lower than that of HA22 (1.2- to 9-fold). The exposure time needed to induce apoptosis in >90% of the cells varied from less than 1 hour in REH, KOPN-8, and CA-46 to more than 4 days in other lines (Fig. 2). For three of the six cells, there was no difference between the activity of LMB-11 and HA22 (SEM, CA-46, and Nalm-6). LMB-11 had to be present for 6 hours on REH and 9 hours on KOPN-8 to induce 90% cell death, whereas HA22 needed less than 1 hour to induce a comparable effect. The pattern was reversed in HAL-01 where LMB-11 induced cell death more effectively and needed less than 24 hours exposure, whereas HA22 had to be present for up to 3 days to induce >90% apoptosis.

Because the finding that CD22-targeting rIT need exposure time to kill ALL cells had not been previously described, we used an *in vivo* approach to confirm the relation of *in vitro* exposure time of KOPN-8 cells to LMB-11 and effective cell killing. Cells were pretreated with 200 ng/ml LMB-11 for various times *in vitro*, washed, and injected into mice to determine if pre-treated cells were alive and capable of engrafting leukemia. Mice injected with untreated KOPN-8 cells lived an average of 25 days (Fig. 3), which was similar to results with 1 to 8 hours of pretreatment (24-27 days). Only if cells were treated for 24 hours prior to injection did mice survive for at least 50 days. These data confirm the findings described in figure 2 that exposure to a high dose of LMB-11 for up to 8 hours was not sufficient to kill all KOPN-8 cells.

The difference in exposure time is related to intracellular rIT processing in KOPN-8 cells

To understand the biological basis of the difference between LMB-11 and HA22 in exposure time needed to kill KOPN-8 cells, we examined several steps in the immunotoxin cell death pathway as recapitulated in Figure 4A (33). To measure rIT uptake into the cell, we labeled the two rITs with Alexa647 and incubated for various times. The rate and amount of uptake was similar for both rITs (Fig. 4B). Once internalized, the catalytic domain is separated from the antibody moiety by furin-cleavage. We analyzed KOPN-8 cell lysates at various times after treatment and found that the furin-cleavage rate differed between the two rITs. There was no cleaved fragment detected for LMB-11 treated cells up to 10 hours (Fig. 4C), whereas HA22 was maximally cleaved at 4 hours after treatment started. The level of cleaved HA22-fragment fell from 6 hours. Because a difference in the amount of furin could explain the difference in rIT-cleavage and therefore rIT-activity, we then tested if the level of total furin correlated with cytotoxicity. By probing for furin levels in a western blot (Fig. 4D) we found that furin levels in all six cell lines were similar. The total furin levels did not

correlate with rIT activity or exposure time needed to kill cells. Because PE stops protein synthesis by ADP-ribosylating elongation factor 2 (EF2), we analyzed the rate of protein synthesis inhibition after treating for various times (Fig. 4E). We found LMB-11 reduced protein synthesis much more slowly than HA22, 66% was inhibited after 8 hours exposure to LMB-11 and after 3 hours exposure to HA22. To correlate arrest in protein synthesis with time required for PE-trafficking and ADP-ribosylation, we assayed EF2 in cell-free extracts. EF2 from rIT-naïve cells can be ADP-ribosylated by rIT, in contrast to EF2 from rIT-treated cells in which protein synthesis had been arrested (34). We lysed cells at various times under treatment, prepared cell-free extracts and ADP-ribosylated the remaining EF2 by adding rIT and biotinylated ADP-ribose in excess. The signal intensity decreased from high in untreated cells to low or absent at later times as more EF2 was modified. In accordance with the [³H]-leucine incorporation data, EF2 from KOPN-8 cells was ADP-ribosylated to more than 75% at 10 hours with LMB-11, whereas it took 4 hours for HA22 to decrease to a comparable level (Fig. 4F). MCL1 levels began to fall accordingly (Fig. 4F). PARP-cleavage was delayed in LMB-11 treated cells. These data indicate that the relative increase of HA22 activity against KOPN-8 cells is due at least in part to more efficient furin-cleavage after internalization, resulting ultimately in a more rapid arrest of protein synthesis and induction of apoptosis.

In vitro* exposure time is a predictor for response *in vivo

We then tested the relevance of LMB-11 exposure time in a KOPN-8 *in vivo* model. The long (9 hours) exposure time required to achieve >90% apoptosis of KOPN-8 cells *in vitro* and the previously demonstrated short (28 minutes) serum half-life of LMB-11 in mice (25) suggested that *in vivo* activity may be limited by inadequate blood concentrations over the required duration to induce maximum cell death. To expose KOPN-8 cells in mice to LMB-11 for various periods of time while keeping the same total dose, we treated mice with either a single dose of 5 mg/kg LMB-11, 2.5 mg/kg given twice 3 hours apart, 1.25 mg/kg given 4 times, 3 hours apart, or with 4 injections of 0.42 mg/kg 3 hours apart, repeated for three consecutive days. Murine BM was analyzed on the day of treatment initiation (Day 8) to define baseline infiltration and 3 days later to evaluate response. The initial BM-infiltration was 3% (Fig. 3B). The 5 mg/kg single-dose, 2.5 mg/kg two-dose, or 1.25 mg/kg four-dose regimens did not result in a reduction of KOPN-8 infiltration. Replenishing blood levels by repeatedly administering LMB-11 four times daily for 3 days reduced BM infiltration 127-fold compared to the day of treatment initiation ($p < 0.001$). Thus, maintaining LMB-11 serum levels at a sufficient concentration over a prolonged period of time without increasing the total dose, markedly improved response with no apparent increase in toxicity.

Because the *in vitro* data showed that HA22 acted much faster in killing KOPN-8 than LMB-11, we hypothesized that bolus HA22 might be very effective *in vivo*. We treated mice from day 8 with three rIT-doses QOD and analyzed for bone marrow infiltration three days later. Vehicle-treated mice showed an infiltration of 6% at initiation of treatment (Fig. 3C), which rose to 58.5% within 1 week. In mice treated with 2.5 mg/kg LMB-11 QOD, KOPN-8 cells grew to occupy 24% of the BM (23,969/10⁵ events, range [21,734-28,984]). In contrast, in mice treated with three doses of 0.4 mg/kg of the rapidly acting HA22, BM

infiltration on day 11 was reduced strongly compared to day 8 ($p<0.0001$) to an average of 0.006% KOPN-8 cells (5/100,000 events, range [2-12]). To assess the impact on survival, we treated mice with the same schedule and followed them until disease progression. Vehicle injected mice survived 21.5 days (Fig. 3D), mice treated with three doses of LMB-11 survived 28 days and HA22-treated mice survived an average of 40 days.

To verify that exposure time is critical to treatment responses, we established a second mouse model with HAL-01 cells, which need longer exposure to HA22 than LMB-11 to be killed in culture (Fig. 2). We found that LMB-11 in bolus doses QOD significantly retarded the growth of HAL-01 compared to vehicle treated mice ($p=0.007$); HA22 was less effective than LMB-11 ($p<0.0001$) (Fig. 3E). Because HAL-01 cells in the BM were growing even though the mice were treated with HA22, we examined our exposure time hypothesis using HA22. To increase the exposure time of HAL-01 cells in the BM to HA22, we used osmotic pumps placed in the peritoneal cavity that delivered HA22 at 0.5 $\mu\text{g}/\text{hour}$ (12 $\mu\text{g}/\text{day}$) continuously over 7 days. The BM-infiltration at treatment initiation on day 8 was 0.55% and rose to 89% within 1 week (Fig. 3E). Mice treated with HA22 QOD presented with 23% infiltration, which was reduced to 0.18% when HA22 was administered continuously by pump. HA22 delivered by pump was more active than HA22 delivered as three bolus injections ($p<0.0001$). These data provide further evidence that maintenance of rIT serum levels impact response *in vivo*.

***In vitro* activity of rIT against patient-derived ALL is exposure time dependent**

The serum half-life of CD22-targeting rITs in children with ALL is short (less than 2 hours) (19, 23). Our cell line data suggest that the response rates in patients might be improved by increased exposure time. Clinical specimens from patients before rIT treatment are limited. It was shown in previous studies that blasts from patient-derived ALL xenografts maintain the characteristics of the originating primary samples (35). Therefore, we generated a sufficient cell supply for further study by expanding primary samples from eight patients in NSG mice. Using these expanded cells, we confirmed that the longer the patient-derived cells were exposed to rIT, the more cells died due to the treatment (Fig. 5A and B). Because these patients were treated in a clinical trial with HA22, we could correlate *in vitro* activity with clinical responses to rIT treatment. Blasts derived from the patient who achieved a complete response needed only a short exposure time to 2.8 nM HA22 *in vitro* for cytotoxicity. Samples from two other patients had a fast response to 2.8 nM HA22 *in vitro*; both showed clinically significant hematologic activity (defined as at least a 50% reduction in blasts and/or normalization of peripheral white blood cell and/or platelet counts). The blasts most resistant to cytotoxicity in this *in vitro* assay were derived from patients who had demonstrated minimal to no response to rIT (stable or progressive disease). As found for KOPN-8 and REH, some patient samples responded faster to HA22 than to LMB-11, while the exposure time needed to induce cell death in the slower responding cells was similar for LMB-11 and HA22. Clinical response of two patients (#4, 5) did not correlate well with *in vitro* cytotoxicity.

Discussion

We report that various CD22-expressing ALL cell lines need to be exposed to rIT for very different time periods to achieve maximal cytotoxicity; the time varied widely from 30 minutes to more than 4 days. This is in contrast to the current opinion that maximal cytotoxic activity correlates with peak concentration. Based on the high number of genetic alterations in ALL, the variability in treatment responses observed was expected (36). We additionally show that the response for LMB-11, a new rIT engineered to have less immunogenicity and reduced non-specific toxicity, is very different from HA22, now in clinical trials. The variation in exposure time has important clinical implications. In clinical trials, rITs are currently given by bolus injection and blood levels fall quickly to sub-therapeutic levels (19, 20, 37). Only cells that are killed rapidly would be expected to respond to such rIT treatment.

The main determinant in the time required to initiate cell death is how quickly and effectively rIT molecules are internalized and traverse the various compartments needed to reach the cytosol and inactivate EF2. In clinical studies, no correlation was apparent between CD22-surface levels and activity of CD22-targeted treatment (38, 39). We found that the rate of furin-cleavage was mainly responsible for the difference in rIT activity in KOPN-8. As recently described (40), furin-cleavage is not mandatory for cell killing by CD22-targeting rITs. However, non-cleavable anti-CD22-rITs show reduced activity, whereas activity can be increased by designing a more efficiently furin-cleaved linker (40). It is therefore possible that LMB-11 is not, or is only poorly, cleaved. The reduced cleavage of LMB-11 likely results in a lower fraction of internalized PE molecules being effectively transported to the cytosol. LMB-11 would be expected to require longer exposure times in comparison to HA22 for a similar amount of PE molecules to reach the cytosol. To identify which of the three differences between LMB-11 and HA22 were responsible for the loss in activity against KOPN-8 (1) F_{ab} instead of dsF_v , 2) deletion of domain II, and 3) 7 mutations in domain III), we produced and tested a total of five proteins (Supplementary Fig. 3). We found that every new component including size, the B-cell epitope depleting mutations and the truncated domain II partially reduced activity and increased the needed exposure time. Further studies to understand the complex biology of these rITs are ongoing.

In mouse experiments with KOPN-8 cells, just three doses of the fast acting HA22 resulted in a near minimal residual disease-negative marrow. LMB-11 only arrested leukemia growth transiently. Response to LMB-11 was improved to a 2-log_{10} tumor burden reduction when the dosing schedule was changed to frequent small doses. As with KOPN-8, bolus injection of LMB-11 only slowed HAL-01 progression, but when administered continuously the HAL-01 leukemic burden was reduced significantly. All animal experiments are in accord with the results of *in vitro* exposure time assays, emphasizing a close correlation between needed exposure times *in vitro* and associated *in vivo* responses. This correlation might be useful for predicting patient responses *a priori*, which is suggested by the trend observed in patient-derived xenograft samples.

Studying response to rIT treatment in our newly characterized systemic ALL xenograft models, we found that the 5 in 100,000 remaining KOPN-8 cells after treatment with HA22

only resulted in extending survival by 18 days. That 0.005% ALL would replace murine BM completely within only 16 doublings (assuming no other organ site of infiltration) explains the relatively short gain in survival. The few surviving KOPN-8 cells suggest a potential reservoir of protected or resistant cells. By combining HA22 with a cytotoxic drug, rIT efficacy might be improved as has been observed in other models (31, 41).

To determine the relevance of our findings, we analyzed primary patient samples from children who had been treated with HA22. Apoptosis was induced rapidly by HA22 in three patients, in three apoptosis was induced slowly and two were resistant. The patient who achieved a complete response had rapid *in vitro* cytotoxicity, as did two other patients with clinically significant hematologic activity. The three patients with the poorest clinical responses had the lowest amount of *in vitro* cytotoxicity at 24 hours. Samples from two patients with clinical activity had intermediate *in vitro* cytotoxicity. Importantly, conclusions from the few cases studied have to be drawn cautiously. Patient-derived ALL samples propagated in NSG mice can maintain original characteristics (35). However, the propagation of such samples in a murine bone marrow microenvironment could introduce bias in the cell populations studied (42). There are likely many additional confounding variables in regard to the multiply relapsed and chemotherapy refractory ALL patient cohort from whom samples were obtained for these studies. For example, clonal heterogeneity could result in engraftment of blast subclones with distinct sensitivity to HA22 in contrast to the clones that originally predominated in patients. Although anecdotal, the data from clinical samples support that exposure time might be relevant for ALL in patients and suggests that increasing exposure to rIT might improve clinical outcomes. Whether our newly developed co-culture assay for rIT activity can be used to predict patient responses *a priori* must be tested prospectively. To what extent our results from pediatric ALL can be applied to rIT activity in adult ALL will be addressed in future studies.

Altogether, two cell lines and three patient samples were killed faster by HA22 than LMB-11; in most of the other samples, cytotoxicity of HA22 or LMB-11 occurred similarly fast. Because ALL patients are immune-suppressed due to disease and treatment, the formation of rIT-neutralizing antibodies is observed less often than in patients with solid tumors (19). Therefore, the higher efficacy of HA22 might be more important than the reduced immunogenicity of LMB-11 when treating patients with ALL. In clinical studies where CD22-targeting rITs were given as IV-bolus injections, the dose limiting side effects were capillary leak syndromes or hemolytic uremic syndromes (23). Whether side effects will be reduced by lower rIT blood levels given over a prolonged period of time will have to be addressed in a dose escalation study. We were not able to assess the MTD of continuous infusion by pump in our animal model because rITs begin to aggregate at higher concentrations. Because the seven times higher total dose of 8.4 mg/kg HA22 by 7-day pump (1.2 mg/kg/day) compared to the MTD of HA22 of 0.4 mg/kg/QOD given 3 times IV (1.2 mg/kg total) was well tolerated, we conclude that toxicity in mice is not increased when this rIT is administered by continuous infusion IP than by bolus IV.

In summary, we find that the rIT exposure time required to kill ALL cells varies widely and that treatment schedules that maintain blood levels for a prolonged period are likely to improve clinical response rates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

B-lineage hematologic malignancies are an important cause of cancer-related mortality and new treatments are needed to overcome drug resistance and reduce nonspecific toxicities. CD22 emerged as an attractive target for antibody-based therapies. HA22 is a CD22-targeting recombinant immunotoxin that is active in children and adults. This study provides insights into the clinically observed variable activity of HA22 against acute lymphoblastic leukemia (ALL). We describe a wide variability of the immunotoxin exposure time on ALL cells needed to reach maximal activity (30 minutes to 4 days). Because of a short serum half-life, *in vitro* exposure time correlated closely with *in vivo* responses in xenograft models. These data suggest that response rates in patients with ALL might be increased substantially by continuous infusion administration. The findings add an important, previously unrecognized exposure time component to the current understanding of a CD22-targeted therapy that may be of relevance to other antibody-based therapies.

Figure 1.

LMB-11 with poor response *in vivo*. A model of HA22 (**A**) which consists of a dsF_v, domain II, and wild-type domain III and LMB-11 (**B**), consisting of an F_{ab}, a furin cleavable linker instead of domain II and the B-cell epitope diminished domain III with mutated amino acids shown as spheres. **C**. NSG mice were injected with KOPN-8 on day 1. Vehicle treated mice were euthanized on days 8, 12, 16, and 20 and analyzed for KOPN-8 BM infiltration. The treatment group received five doses of 2.5 mg/kg LMB-11 IV on days 8, 10, 12, 14, and 16. Mice were euthanized two days after each time point. Values are averages of three mice, errors as SD, p-value determined by two-way ANOVA. **D**. KOPN-8 cells were treated *in vitro* for 1 hour with indicated concentrations of LMB-11-Alexa647, not internalized rIT was washed away with 0.2 M Glycine pH 2.5 and MFI determined by flow cytometry. Absolute molecule numbers were generated using Alexa647-beads. Mice were injected with 2.5 mg/kg LMB-11-Alexa647, euthanized 1 hour after injection and analyzed for Alexa647-signal intensity. Each value shown is an average from three independent animals, error as SEM. *Dashed line indicates an average of 20,500 molecules internalized by the KOPN-8 cells in murine BM, correlating to 200 ng/ml LMB-11 *in vitro*. †Dotted line marks *in vitro* IC₅₀ of LMB-11 (0.8 ng/ml) correlating to 220 LMB-11 molecules.

Figure 2.

Cell- and rIT-specific exposure time needed to induce cell death. Cells were treated with 2.8 nM rIT for indicated times, washed, and re-plated in medium without rIT. Seventy-two hours after assay initiation, cells were washed and stained with Annexin-V PE and 7-AAD. Percentage Annexin-V and 7AAD negative cells are plotted over exposure time. The bar graphs show a representative of at least three similar results.

Figure 3.

In vitro determined exposure time predicts *in vivo* response. **A.** KOPN-8 cells were left untreated or treated with 2.8 nM LMB-11 for indicated times, washed twice with PBS and five million cells of each set were injected per mouse. Mice were euthanized at >10% weight loss. **B.** KOPN-8 cells were injected in NSG mice on day 1. Untreated mice were euthanized on day 8. Five mice per group were treated with 100 µg of LMB-11 (5 mg/kg) given either as one single dose on day 8, two consecutive doses of 50 µg every 3 hours on day 8, four consecutive doses of 25 µg given every 3 hours on day 8, or four consecutive doses of 8.3 µg every 3 hours on days 8, 9, and 10. All treated mice were euthanized on day 11 and %-KOPN-8 BM infiltration was determined by flow cytometry. **C.** KOPN-8 cells were injected IV on day 1. Some mice were euthanized on Day 8 to define tumor burden at treatment initiation or treated three times with either vehicle, 2.5 mg/kg LMB-11, or 0.4 mg/kg HA22 on days 8, 10, and 12. All mice were euthanized on day 14 and BM was analyzed for KOPN-8 infiltration by flow. Shown is the mean percentage of remaining KOPN-8 infiltration. **D.** Mice were injected with KOPN-8 on Day 1 and with rITs on days 8, 10, and 12 similar to **A.** Animals were euthanized at >10% weight loss. P-values were determined by log-rank test. **E.** Mice were inoculated with 2 million HAL-01 cells on day 1. On day 8, mice were either treated IV with vehicle, 3 x QOD with 2.5 mg/kg LMB-11, or 0.4 mg/kg HA22, or mice received an ALZET osmotic pump implant IP which injected 0.5 µg/hour HA22 into the peritoneal cavity for 7 days. **B, C,** and **E,** bars indicate mean, symbols show individual mice, error is shown in SD, significance levels for two group comparisons in **B, C,** and **E** were determined by unpaired t-test as ns = not significant, * p<0.05, ** p<0.1, *** p<0.001, **** p<0.0001.

Figure 4.

Differences in exposure time for KOPN-8 are related to intracellular rIT processing. **A.** Cartoon describing rIT pathway, as modified from (33). rIT is internalized after receptor binding, furin separates the antibody fragment from PE, which then traverses the ER via retrograde trafficking, enters the cytosol where it ADP-ribosylates EF2 (ADPr-EF2), which arrests protein synthesis. This results in a fall of MCL1 followed by a destabilization of mitochondria and activation of intrinsic apoptosis. **B.** KOPN-8 cells were incubated with Alexa647-labeled rIT for indicated times. Surface bound molecules were washed off in 0.2 M Glycine pH 2.5 and signal intensity for internalized molecules determined by flow. Each time point represents biological triplicates. Shown is the average of three independent experiments. P-value for difference was determined by 2-way ANOVA. **C.** One million KOPN-8 cells were treated with 200 ng/ml LMB-11 or equimolar HA22 for indicated times or left untreated. Cells were lysed after the indicated time, separated by SDS-PAGE under reducing conditions, blotted, probed for with polyclonal rabbit anti-PE, and detected with anti-rabbit-HRP. Expected height for whole (wh) HA22-Vh-PE38 (51 kDa), furin-cleaved (cl) HA22 fragment (34 kDa) or whole (50 kDa) and cleaved (25 kDa) LMB-11-Vh-PE24 are indicated. Shown is a representative of four independent experiments with similar results. **D.** One million cells of each cell line were lysed, similar volumes separated by SDS-PAGE, and analyzed by western blot. Shown is a representative blot and average densitometry values from four independent experiments. Error shown as SD. Values were background subtracted (net value) and normalized to actin loading control (net furin/net actin). **E.** KOPN-8 cells were incubated with 2.8 nM of the respective rIT for indicated times, then ^3H -leucine was added, incubated for an additional hour and analyzed for ^3H -

leucine incorporation. Counts per minute were normalized to untreated cells after background subtraction. Values are derived from two independent experiments each in triplicate. P-value for difference was determined by 2-way ANOVA. **F.** KOPN-8 cells were treated with 2.8 nM of the indicated rIT various times after which cells were lysed and cell lysates were treated with NAD-biotin and rIT in excess in a cell free reaction to label the remaining unmodified EF2 with biotinylated ADP-ribose, which was then probed by Streptavidin in a western blot (shown as ADP-EF2 signal). The same lysates were used in western blots for MCL1, PARP, and GAPDH. Shown are representatives of at least three biological repeats.

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Figure 5.

Clinical response for HA22 treated patients correlates with *in vitro* rIT exposure time needed to kill ALL blasts. Primary blast samples from patients before HA22 treatment were expanded in NSG mice. These cells were plated on OP9 cells and exposed to 2.8 nM LMB-11 (**A**) or HA22 (**B**) for the indicated times. The clinical response to HA22 is indicated below each respective patient in **B** as CR (complete response), PR (partial response), HA (hematological activity), SD (stable disease), or PD (progressive disease). Cells were analyzed 3 days after assay initiation by flow cytometry. After staining with human CD19 to determine the ALL population cells were analyzed for cell death as determined by Annexin V-PE/7-AAD. The living ALL was >50% in all untreated samples and viability was normalized within each sample as 100% to the highest viability. Shown is a representative set of at least three biological replicates with similar results.

Table 1Toxin IC₅₀ *in vitro* by WST-8

	LMB-11		HA22	
	pM	ng/ml	pM	ng/ml
KOPN-8 (ALL)	10.9	0.8	1.2	0.08
REH (ALL)	2.9	0.2	0.3	0.02
CA-46 (BL)	5.8	0.4	1.5	0.1
HAL-1 (ALL)	25.5	1.9	29.6	1.9
SEM (ALL)	62.8	4.6	50.9	3.2
Nalm-6 (ALL)	54.9	4.0	32	2.0

Values represent averages of at least three independent experiments, each in triplicate.

ALL = acute lymphocytic leukemia; BL = Burkitt's lymphoma