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Genetic Alteration of a bispecific ligand directed toxin targeting human CD19 and CD22 receptors resulting in improved efficacy against systemic B cell malignancy

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Abstract

A bispecific ligand-directed toxin (BLT) called DT2219ARL consisting of two sFv ligands recognizing CD19 and CD22 and catalytic DT390 was genetically enhanced for superior in vivo anti-leukemia activity. Genetic alterations included reverse orienting VH-VL domains and adding aggregation reducing/stabilizing linkers. In vivo, these improvements resulted in previously unseen long-term tumor-free survivors measured in a bioluminescent xenograft imaging model in which the progression of human Raji Burkitt's lymphoma could be tracked in real time and in a Daudi model as well. Studies showed DT2219ARL was potent (IC50s 0.06–0.2 nM range) and selectively blockable. Imaging studies indicated the highly invasive nature of this B cell malignancy model and showed it likely induced preterminal hind limb paralysis because of metastasis to spinal regions prevented by DT2219ARL. DT2219ARL represents a new class of bispecific biological that can be continually improved by genetic mutation.

Keywords

immunotoxin; diphtheria toxin; leukemia; lymphoma; scid model; anti-CD19 sFv; anti-CD22 sFv

INTRODUCTION

Acute leukemia is the most common childhood malignancy, representing 30% of all cancer in American children under the age of 15 years and 12% of cancer cases in those aged 15 to 19 years old. In the United States, approximately 2500 new cases are diagnosed annually; 80% of these are B lineage acute lymphoblastic leukemia (B-ALL). Chemotherapy resistant blasts are a frequent cause of treatment failure in all leukemia patients [1] and alternative therapies are urgently needed. Immunotoxins (IT) are synthesized by coupling an antibody or antibody fragment to a potent, catalytic toxin, such as diphtheria toxin, capable of inhibiting protein synthesis [2]. Catalytic toxins are chosen because one molecule entering the cytosol can kill a cell. For CD19 targeting, investigators using conventional biochemically linked anti-CD19 IT have reported anti-cancer effects [3–7]. However, these have not reached the mainstream

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because of varied degrees of effectiveness. The 95 kDa CD19 membrane glycoprotein is considered by many to be the most ubiquitous marker expressed on B cells. CD19 is expressed not only on mature B cells, but also on late pre-B cells. It is broadly expressed on B cell leukemia/lymphoma [8] including B-ALL.

Anti-CD22 IT have proven successful in the treatment of rare Hairy Cell Leukemia (HCL) [9]. However, HCL represents a narrow sampling of patients with leukemia and expanding the use of the drug to the wider population of patients is critical. In a previous study, we cloned a new molecule by fusing two repeating sFv subunits recognizing human CD19 and human CD22 spliced downstream of truncated DT390 to broaden our toxin delivery and our anti-leukemia affect [10]. Studies targeting these 2 ligands with monomeric conventional immuntoxins showed promise and led to clinical trials [11,12].

Bispecific ligand directed toxins (BLTs) are novel single-chain biologicals synthesized by linking a truncated toxin to two well established targeting ligands with the goal of increasing targeting capability. For successful BLT, the final construct must have better anti-tumor activity than its monospecific counterparts or a mixture of the two, thus indicating an advantage of including both ligands on the same single chain molecule [13–17]. DT2219ARL fulfilled these criteria for a successful BLT.

Diphtheria toxin [DT] was chosen for construction due to its irreversible catalytic activity and research demonstrating a single molecule causes cell death [18]. Also, it is desirable to have new anti-cancer agents that kill by protein synthesis inhibition, a mechanism entirely different and unrelated to the mechanism of most conventional chemotherapeutic agents. The truncated form of DT used in this study (DT390) was selected due to previous research describing a series of internal frame deletion mutations that established amino acid 389 as the best location for genetic fusion of DT to targeting ligands [19]. DT390 contains the A fragment of native DT that catalyzes ADP ribosylation of elongation factor 2 (EF-2) leading to irreversible inhibition of protein synthesis and cell death [20,21].

This study set out to determine whether a BLT could be genetically engineered to improve its ability to target human malignant B cells in vivo. Important changes were made requiring the recloning of the entire target gene. The variant protein called DT2219ARL was tested for its superiority in vitro and its ability to produce long-term cancer free survivors in two highly aggressive scid mouse models of human B cell cancer using in vivo bioluminescence imaging and survival.

MATERIALS AND METHODS

Construction of DT2219EA

For these studies, 3 different variations of DT2219 were synthesized, DT2219EA, DT2219EB1, and DT2219ARL. In 2005, we reported our original DT2219 2,760 bp construct called DT2219EA constructed using DNA shuffling and assembly PCR (10). DT2219ARL consisted of an Nco I restriction site at the n-terminus, followed by a downstream ATG initiation codon, the first 389 amino acids of the DT (DT390), the VH and VL regions of anti-CD22 (sFv) and anti-CD19 [22] linked by a 20 amino acid segment of human muscle aldolase (hma), and a XhoI compatible restriction site (Figure 1). Importantly, Salvatore et al reported that mutating three amino acids Thr-His-Trp (THW) in place of Ser-Ser-Tyr (SSY) at positions 100, 100A, and 100B in the CDR3 region of the VH of the anti-CD22 sFv enhanced its affinity, so these same amino acids were mutated in the assembled plasmid called pDT2219hmaEA.pET21d, which produced DT2219EA (enhanced affinity) [23]. The synthesis of the DT gene fragment was previously described [24] using diphtheria toxin CRM107 as a template. The hma fragment was used as a non-immunogenic linker to connect the two sFvs

and was used to enhance the level of protein production and ultimately the level of purity of the molecule. HMA is 363 amino acids in length and we used the final 20 amino acids (PSGQAGAAASESLFVSNHAY). DNA sequencing analysis (University of Minnesota, Advanced Genetic Analysis Center) was used to verify that the gene had been cloned in frame and was correct in sequence.

Construction of DT2219EB1

DT2219EB1 was created by mutating DT2219EA by modifying two hot spot amino acids (S30G and N31R) in the anti-CD22 VL region as previously reported by Ho et al. [25]. The sequence change was verified.

Construction of DT2219ARL

The hybrid gene encoding DT2219ARL was synthesized using assembly PCR. The major differences between DT2219ARL and DT2219EA were 1) reversal of the orientation of the VH and VL chains. In DT2219ARL, the VL preceded the VH (Figure 1). 2) The VL and VH genes of each sFv were conjoined by a fragment encoding the ARL linker (GSTSGSGKPGSGEGSTKG) and the two sFv genes were linked by a fragment encoding G4S linker. In its final configuration, the DT2219ARL NcoI/XhoI gene fragment encoded a start codon followed first by 389 aa of DT, and then a 7aa linker EASPEEA, followed by the anti-CD22 sFv, and then the CD19 sFv. The final target gene was spliced into pET21d vector expression vector and inclusion bodies expressed. An FDA IND is now approved for the clinical phase I evaluation of DT2219ARL.

As specificity controls for these studies, we constructed a bivalent fusion protein consisting of DT390 fused to two repeating sFvs recognizing human CD3epsilon called Bic3 [26]. Anti-CD3epsilon recognizes a domain of the T cell receptor [27]. An additional control included DT390EpCam23, DT390 spliced to anti-EpCam sFv and anti-ErbB2sFv sFv. Anti-EpCam and anti-ErbB2sFv have been used by others to synthesize recombinant IT [28,29]. ErbB2 is a tumor-associated antigen belonging to the epidermal growth factor receptor family and implicated in poor prognosis and more aggressive course in many human cancers including breast, lung, ovary and stomach [30].

Inclusion body isolation, Refolding and purification

Plasmid was transformed into the Escherichia coli strain BL21(DE3)(EMD, Madison WI). Bacteria were grown in 600 ml Luria Broth supplemented with 100 ug/ml carbenicillin in a 2L flask at 37°C with shaking. Expression of the hybrid gene was induced by the addition of isopropyl-b-D-thiogalactopyranoside (IPTG, FisherBiotech Fair Lawn, NJ). Two hours after induction, the bacteria were harvested by centrifugation. The cell pellets were suspended and homogenized using a polytron homogenizer. After sonication and centrifugation, the pellets were extracted with 0.3% sodium Deoxycholate, 5% Triton X-100, 10% Glycerin, 50 mM Tris, 50 mM NaCl, 5 mM EDTA, pH8.0 and washed.

The proteins were refolded using a sodium N-lauroyl-sarcosine (SLS) air oxidation method modified from a previously reported procedure for isolating sFv (26). Refolded DT2219 was purified by FPLC ion exchange chromatography (Q Sepharose Fast Flow, Sigma, St. Louis, MO) using a continuous gradient from 0.2 M to 0.5 M NaCl in 20 mM Tris-HCl, pH 9.0 over 4 column volumes.

Antibodies and cell lines

The anti-CD19 monoclonal antibody hybridoma HD37 that secretes mouse IgG1 kappa has been previously described by Dorken et al. [31] and has been studied as a targeted toxin

conjugated to ricin toxin A chain [32]. RFB4 (anti-CD22) was provided by Dr. Ellen Vitetta, University of Texas Southwestern Medical Center, Dallas TX. Anti-Ly5.2, a rat IgG2a from clone A20-1.7, generously provided by Dr. Uli Hammerling, Sloan Kettering Cancer Research Center, New York, NY. Anti-Ly5.2 was used as a control since it recognized mouse CD45.1, a hematopoietic cell surface marker not expressed on human cells.

Human cell lines included the CD19–CD22– T cell leukemia HPBMLT [33] and the CD22 +19+ Burkitt's lymphomas Daudi [34] and Raji [35]. Raji was genetically altered by transfection with dual reporter genes encoding both firefly luciferase and GFP creating the Raji-luc cell line for imaging. Raji-luc was subcloned using flow cytometric cell sorting in order to obtain stable transfectants that were highly bioluminescent.

Measuring DT2219ARL activity in vitro. To determine the effect of DT2219 on normal B and malignant B cell function, the Daudi CD19+CD22+ Burkitt's lymphoma cell line was used. Flow cytometry shows that Daudi is >98% positive for both CD19 expression and CD22 expression (data not shown). Cells (105) were plated in a 96-well flat-bottom plate in RPMI supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin. Immunotoxin in varying concentrations was added to triplicate wells containing cells. The plates were incubated at 37°C, 5% CO₂ for 72 hr. Cells were then incubated with one μ Ci [methyl-3H]-thymidine (GE Healthcare, UK) per well for eight hours and harvested onto glass fiber filters, washed, dried and counted for ten minutes in a standard scintillation counter. Data were analyzed using Prism 4 (GraphPad Software, Inc) and were presented as “percent control response” calculated by dividing the cpm of untreated cells by the cpm of the immunotoxin- treated cells ($\times 100$).

Blocking studies were conducted to test the specificity of DT2219ARL. Briefly, 0.5, 5, 50, or 500 nM RFB4 or HD37 were added to media containing 10 nM DT2219EA, DT2219EB1, or DT2219ARL. Resulting mixtures were added to wells containing Daudi cells and proliferation was measured by 3H-thymidine uptake as described. The mouse specific antibody Ly 5.2 was studied as a negative control. Data were presented as “percent control response” as described above.

Flow Cytometry Studies—To determine comparative Kds, DT2219ARL-FITC and control Bic3–FITC and DTe23EpCAM-FITC were reacted with Daudi cells, Raji cells, normal human peripheral blood mononuclear cells (PBMC), normal human CD22+, magnetic bead enriched PBMC, and patient CLL cells. Cells were incubated with recombinant FITC-labeled proteins at saturating concentrations for 45 minutes at 4° C. Positive cells were quantitated using a Becton Dickinson FACS Calibur. Kds were calculated using PRISM software. DT2219ARL-FITC reactivity was also determined with non-human primate cells. Rhesus monkey PBMC cells were obtained through RAR, University of Minnesota. As controls, binding was simultaneously assessed against human PBMC and human CD22+, magnetic bead enriched PBMC enriched using a CD22 isolation kit and a MACS system (Miltenyi Biotec, Auburn, CA).

Mouse efficacy studies—Female scid/hu mice were purchased from NCI, Frederick Cancer Research and Development Center, Animal Production Area and housed in an AAALAC-accredited specific pathogen-free facility under the care of the Department of Research Animal Resources, University of Minnesota. Animal research protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC). Animals were housed in microisolator cages to minimize the possibility of transmission of any contaminating virus.

Experiment 1: For experiment 1, 106 Daudi cells in 200 μ l sterile, endotoxin-free PBS were injected intravenously into scid mice via caudal vein. After Daudi injection on day 0, one 20 μ g IV injection of DT2219ARL or DT2219EB1 was given on day 3 with five subsequent 10 μ g/200 μ l IP injections on days 5, 10, 24, 26 and 31. Body weights were documented three times per week. Since this Daudi substrain always metastasizes to the central nervous system resulting in hind-limb paralysis (HLP), paralyzed mice were deemed pre-terminal and euthanized by University approved IACUC procedures.

Experiment 2: Mice were given 106 Daudi cells IV on day 0 and divided into groups. Groups of 7 mice (no treatment group, n=5) were given IP injections of 20 μ g/200 μ l DT2219ARL, DT2219EA, or Bic3 on days 3 and 6; 10 μ g/200 μ l IP injections on days 21, 24, 26, 31, 38, 47, 54, 56 and 59.

Experiment 3: Mice were given 106 Daudi cells IV on day 0. A single 20 μ g IP injection of DT2219ARL or DT2219EA was given on day 3. Body weights were determined. Mice exhibiting hind limb paralysis were euthanized.

Experiment 4: In order to test the efficacy of DT2219ARL against a different CD22+CD19+ human B cell malignancy, mice were given 106 Raji-luc cells IV on day 0 and then were treated with a single 20 μ g IP injection of drug on days 3, 5, 11, 16, and 18. Mice were imaged on day 5, 12, 18, 25, 32, 39, 46, 60, 74, and 87. Images were captured using Xenogen Ivis imaging system (Xenogen Corporation, Hopkington MA) and analyzed with IGOR Pro 4.09a software (WaveMetrics, Inc., Portland, OR). Prior to imaging, mice were anesthetized using isoflurane gas. All mice received 100 μ l of a 30 mg/ml D-luciferin aqueous solution (Gold Biotechnology, St. Louis MO) as a substrate for luciferase 10 minutes before imaging. All images represent a 5 minute exposure time and all regions of interest (ROI) are expressed in units of photons/sec/cm²/sr.

Statistical analyses

Groupwise comparisons of continuous data were made by Student's t-test. A computer program for compiling life table and statistical analysis by the Log-Rank test was used to analyze survival data. Probability (p) values < 0.05 were considered significant.

Rabbit studies

Female New Zealand White rabbits (2.2 kg) were purchased from Bakkom Rabbitry (Viroqua, WI) and housed under the care of Resource Animal Resources as described above. Catheters were implanted in the ears for IV drug administration. Animals were weighed to determine the effect of drug on weight. Blood samples were obtained centrifuged immediately at 5,000 RPM. Individual serum samples were analyzed on a Kodak ETA-CHEM 950 by the Clinical Chemistry Laboratory, University of Minnesota Hospital, Fairview (Minneapolis, MN). The BUN assays were read spectrophotometrically at 670 nm. In the ALT assay, the oxidation of NADH was used to measure ALT activity at 340 nm.

RESULTS

Purification of DT2219ARL

Following ion exchange chromatography, 95 kDa DT2219ARL, DT2219EA, and DT2219EB1 were greater than 95% pure as determined by Coomassie blue staining (Figure 1B).

Cytotoxicity of various IT on the Daudi Cancer Cell Line

Daudi was selected as a target cell line in these studies because flow cytometry studies showed greater than 95% positivity for both CD19 and CD22 (not shown). To determine the ability of DT2219ARL, DT2219EA, or DT2219EB1 to kill Daudi, these IT were tested in a proliferation assay and a representative experiment is shown (Figure 2A). DT2219ARL showed an IC₅₀ of 0.2 nM. DT2219EA showed an IC₅₀ of 0.4 nM. DT2219EB1 showed an IC₅₀ of 0.1 nM. None of these curves statistically differed. Figure 2B shows a different experiment in which DT2219ARL had no effect on CD22-CD1-HPBMLT T leukemia cells. In contrast, HPBMLT were readily killed with an anti-T cell IT called Bic3. To create the mutant DT2219GE gene, the DT2219 gene was disrupted by a single glycine to aspartic acid mutation at position 53 of the DT390 molecule known to inactivate the catalytic activity of the DT A chain. Whereas parental DT2219ARL showed an IC₅₀ of 0.06 nM, the mutated DT2219GE protein minimally inhibited Daudi proliferation. Together, these data showed that DT2219 variants were potent and selective in their ability to inhibit CD22+CD19+ target cells and that the killing of DT2219 IT is caused by the DT moiety, not the 2219 moiety (Figure 2C). Trypan blue viability assays were performed in addition to proliferation assays and as an additional check to verify that DT2219ARL was indeed killing and not simply inhibiting cell proliferation/protein synthesis.

Increased activity of DT2219ARL is due to the presence of the anti-CD22 and anti-CD19 sFv ligands on a single molecule

Proliferation assays were conducted in order to determine if the increased activity of DT2219ARL was a result of the increased number of binding molecules present on a bispecific IT. Figure 2D shows the data comparing the activity DT2219ARL to the monospecific DT22 and DT19, as well as a combination of both monospecific IT against Daudi cells. A mixture of DT22 and DT19 resulted in an identical number of ligands as are present in the same concentration of DT2219ARL. Against Daudi, the monospecific DT22 was able to kill with an IC₅₀ of 3.05 nM. Monospecific DT19 was less effective. However, DT2219ARL showed an IC₅₀ of 0.15 nM, representing about a 1000-fold increase in activity as compared to DT19 and a 20-fold increase in activity as compared to DT22. Interestingly, a mixture of DT22 and DT19 showed no increased in activity over DT22 alone. These data demonstrate the superior activity of DT2219ARL is due to the presence of both ligands on a single-chain molecule.

Blocking DT2219 activity

To confirm that the anti-CD19 sFv and anti-CD22 sFv ligands were both still active in DT2219, blocking experiments were performed with the parental RFB4 and HD37 monoclonal antibodies. Proliferation experiments were performed in which increasing amounts of blocking antibody were added to a constant inhibitory concentration of 10 nM DT2219 immunotoxin which inhibited about 90% of Daudi cell proliferation (IC₉₀). Figure 3A shows that increasing concentrations of RFB4 or HD37 inhibit the proliferation of 10 nM DT2219ARL in a dose-dependent manner. Saturation is reached around 50 nM. The addition of an irrelevant control antibody anti-Ly5.2 had no effect. Neither antibody blocked 100% of the activity because blocking one ligand would not necessarily fully block the other. Similar results were observed when DT2219EA or DT2219EB1 were blocked in an identical fashion (Figure 3B and 3C). None of the antibodies alone were stimulatory to Daudi cells at these concentrations. Together, the similarity of these curves indicated that the anti-CD19 and anti-CD22 ligands on the DT2219 variants appeared to bind with similar monovalent affinity. Also, both sFvs were active on the DT2219 molecules which were highly specific.

Flow cytometry results

Briefly, Table 1 shows that DT2219ARL-FITC was highly reactive with normal human B cells in the form of PBMC with a K_d of 28 nM. For these studies, we gated the 7% B cells found in

normal human peripheral blood. These findings were confirmed by testing DT2219ARL against magnetic bead enriched CD22+CD19+ B cells (enriched to 90%). The K_d of the enriched cells was similar at 20 nM. When DT2219ARL was tested against human malignant B cell lines in the form of the Daudi and Raji cell lines, the K_d of Daudi was 133 nM and Raji was 39 nM. These findings indicate that we can anticipate different K_ds on different cell lines, some lower than others. Finally, we tested DT2219ARL binding against human malignant B cells in the form of peripheral blood B-CLL from two different patients. Patient 1 had a K_d of 36, while patient 2 had a K_d of 181. Together, these data indicate that DT2219ARL will bind malignant B cells from patients, but we can anticipate patient-to-patient variation, perhaps due to variances in CD22 and CD19 expression levels. As a negative control we tested the binding of irrelevant immunotoxins that do not bind to human B cells, DTEpCam23-FITC and Bic3-FITC. They showed a K_d of 1,879 and 1,032 nM, respectively indicating that the binding of DT2219ARL is specific. These data suggest that there is no major difference in the binding of DT2219ARL to normal and malignant B cells and that binding is specific.

Figure 4 compares the reactivity of DT2219ARL-FITC with human PBMC, monkey PBMC, and magnetic bead enriched CD22+ human PBMC. The top 3 panels show that CD22 enriched human PBMC were highly reactive (79.1%) with a saturating concentration of DT2219ARL-FITC (100 nM). A negative control DTEpCam23-FITC was not as reactive with human CD22 + enriched PBMC. A control conventional anti-CD22-FITC antibody (RFB4) was highly reactive. In the same experiment, the lowest 3 panels show that DT2219ARL-FITC did not recognize monkey PBMC (2.28%), even though the positive control anti-CD22 antibody did recognize them (31.3%). The parental anti-CD19 antibody HD37 did not recognize monkey cells. The middle 3 panels showed that DT2219ARL-FITC did recognize human PBMC. The parental anti-CD22 and anti-CD19 monoclonal antibodies also recognized B cells in the peripheral blood. Note that there are considerably less positive B cells because these are PBMC and not a CD22-enriched population.

Effects of DT2219 IT in SCID mice with systemic cancer

Injection of the Daudi cells intravenously into scid mice results in a systemic tumor that infiltrates all major organs and is reminiscent of human leukemia. To determine if DT2219ARL was effective against established systemic leukemia and whether it differed in its effectiveness from DT2219EB1, systemic cancer was initiated in mice and ip treatments were started on day 3. Figure 5A shows that mice given 6 ip injections of DT2219ARL survived significantly longer than mice given treatment with DT2219EB1 or untreated mice ($p < 0.001$). All of the DT2219ARL treated mice survived to day 90 when the experiments were terminated. All of the untreated control mice and the DT2219EB1 treated mice were dead by day 90. Three of five mice given DT2219EB1 died early by day 10 with weight loss (not shown) indicating that DT2219EB1 is more toxic than DT2219ARL.

Figure 5B shows a different experiment in which mice were given 11 injections of DT2219ARL. Again, all of these mice survived 150 days compared to groups of mice treated with control BIC3 or untreated controls ($p < 0.001$). DT2219EA also showed enhanced survival, but over 60% of these mice were dead by day 80. No early toxic deaths resulted from DT2219EA or DT2219ARL treatment. Figure 5C shows that when mice are given a single dose of DT2219ARL survival is significantly better than untreated controls ($p < 0.01$). DT2219EA also shows a protective effect that is not as pronounced as the effect with DT2219ARL.

To study a second human B cell malignancy in a model which could be imaged in real time, Raji-luc was injected intravenously into scids to induce systemic cancer. Figure 6A shows that tumor progressed quickly since it was detected in all untreated mice ($n=4/\text{group}$) by day 12–18 and all untreated mice developed hind limb paralysis on days 18 through 46. Animals

injected with Raji-luc develop this CNS complication with a 100% incidence. Three of four (75%) of the DT2219ARL treated mice were completely tumor free on day 87. Tumor progressed in one of the treated mice on day 18. Figure 6B shows the total photon activity graphed over time for each mouse. In Figure 6C, three additional mice were injected with Raji-luc and not treated in order to study the aggressive nature of the tumor with our dual reporter gene cell line. Luciferase bioluminescent imaging showed these animals all developed tumor by day 14. GFP imaging of the same mice on day 21 confirmed tumor presence in lung and immune system and revealed the tumor had a propensity for the bone marrow and spinal cord.

Together, these data indicate that DT2219ARL was able to prevent the onset of established fatal systemic cancer in two highly aggressive human B cell malignancy models in scid mice.

Effects of DT2219ARL in rabbits

To determine the MTD of DT2219ARL, rabbits were injected with a course of 4 IV injections given every other day. Rabbits were given either 50, 100, 200, or 500 ug/kg. Two animals were treated at each dosage. Figure 7A shows the average weight loss and reveals a minimal effect at 50 or 100 ug/kg treatment as compared to the untreated controls. At 200 ug/kg there was a more pronounced weight loss amounting to less than 20% of starting weight that is not considered life-threatening by our IACUC. Weight loss is likely due to non-specific toxicities of the agent, becoming obvious at higher doses. These are likely due to non-specific uptake of DT390, primarily causing liver damage.

BUN levels correlated with increasing concentrations of DT2219ARL with only the 500 ug/kg dosage causing elevations. This increase only reached 100 mg/dL and was considered minimal and histology confirmed mild effects in the kidneys (not shown). In contrast, DT2219ARL was highly damaging to liver at 500 ug/kg, but not at 200 ug/kg. Not only did these animals lose weight, but Figure 7B shows a precipitous rise in ALT levels indicating dose-dependent hepatic damage. Damage was confirmed by histology studies which showed necrosis and fatty degeneration consistent with grade 4 liver damage (Figure 7C). Together, the ALT, histology, and weight data confirmed that the dose dependent toxicity of DT2219ARL was likely due to non-specific uptake of DT390, primarily causing liver damage. The 500 hundred ug/kg dosage damaged the liver and the MTD was 200 ug/kg. Histology studies of the heart, lung, and spleen did not show any evidence of cellular damage or toxicity.

DISCUSSION

The major original findings of this study are: 1) mutagenesis of DT2219 [10] resulted in a new drug DT2219ARL with highly improved systemic efficacy. 2) The new variant significantly prevented the onset of established fatal systemic cancer in two highly aggressive human B cell malignancy models in scid mice. The use of a dual monitoring optical modeling system provided important information for tracking metastases to organs. 3) The dose limiting toxicity of the drug in rabbit studies was hepatotoxicity and a safe maximal tolerated dose was 200 ug/kg. 4) In vitro studies show that the bispecific DT2219ARL had greater activity than its two monospecific counterparts DT22 and DT19 and that the presence of both ligands on the same bispecific molecule is responsible for the superior activity of DT2219ARL. Thus, including two well-established targeting ligands, anti-CD22 and anti-CD19 on the same single chain molecule has distinct advantages over the same monospecific targeting agents.

In this paper, three different variations of DT2219 were synthesized and tested: DT2219EA, DT2219EB1, and DT2219ARL. DT2219EA was previously reported and its most notable features are the VH-VL orientation of the variable chains, a 20 amino acid segment of hma connecting the two sFvs, and the mutation of three amino acids in the CDR3 region of the VH domain of the anti-CD22 sFv which enhanced its affinity. Since studies with anti-CD22 sFv

show that enhanced affinity might further benefit the tumor localization of our construct [10], DT2219EB1 was synthesized. This variant was identical to DT2219EA, except for a modification of three additional “hot spot” amino acids in the anti-CD22 VH heavy region that further enhanced affinity [23]. The third construct was DT2219ARL and its most notable features were reversed orientation of the VH and VL chains, and the fact that the VL and VH genes of each sFv were conjoined by a fragment encoding the ARL linker, and the hma linker between the 2 sFv was replaced with a single Gly4Ser linker. The hma linkers were replaced because of concerns that the larger hma linkers originally added to DT2219EA to enhance protein yield, might disrupt the 3D VH-VL structure enough to negatively impact DT2219EA activity which would explain our lack of activity in vivo. The ARL linkers were included to solve scale-up issues since charged residues within ARL include 2 positively charged lysines and a negatively charged glutamic acid which improved refolding and helped increase yields beyond those obtained with constructs made without these sequences [26]. Any or all of these changes may have significantly contributed to the gain in DT2219ARL functionality.

Although DT2219EA did significantly delay the onset of lethal Daudi infection in scids in previous studies, it did not produce long-term tumor free survivors despite multiple ip injections (10). In contrast, DT2219ARL did produce long-term cancer free survivors using 2 different human B cell malignancies, Daudi and Raji. In this paper, when DT2219ARL was directly compared to DT2219EA, it was significantly better and bioluminescent imaging was used to verify that animals were cancer free in the Raji-luc model. The advantages of dual imaging system was that luciferase imaging was able to track tumor growth throughout the animals, verify the extremely aggressive nature of Raji-luc, and showed that despite rapid and aggressive onset, limited treatment was sufficient to produce long-term tumor free survivors. On the other hand, GFP imaging generated finer images that showed tumor not only grew in immune tissue and lung, but also had a propensity for bone marrow and spinal cord. The GFP images indicate that compression of the spinal cord explains why all of these animals develop CNS effects and hind limb paralysis immediately before their death, an observation not possible with luciferase imaging by itself. The data indicate that this model will be extremely valuable for tracking tumor progression and metastasis in mice that fail therapy.

Mechanistically, DT2219ARL could be superior because of better binding, internalization, or intra-cellular trafficking. If we target CD19 and CD22 simultaneously with DT2219ARL, then the total density of antigens seen by DT2219ARL is the sum of the CD19 and CD22 densities, which would be greater than the CD22 density alone or the CD19 density alone. However, a mixture of monospecific DT19 and DT22 did not improve activity. Thus, enhanced binding is not the sole explanation. Recent studies by others comparing anti-CD22 and anti-CD19 IT showed that anti-CD22 IT was internalized better than anti-CD19 IT [36]. This occurs despite the fact that CD19 densities on B cell malignancies often are greater than CD22 densities [37]. The inclusion of anti-CD19sFv on the same molecule as anti-CD22 might facilitate the amount of IT that gains cell entry through membrane clathrin coated pits [38]. Furthermore, the inclusion of anti-CD19 might impact the rate of entry of DT to critical cell organelles such as lysosomes or ER. For example, earlier studies showed that different IT ligands (such as CD2, CD5, and CD3) affected the rate of entry of IT into the lysosomal compartment [39]. Creating bispecific IT may be a means to improve IT internalization and trafficking.

Perhaps DT2219ARL has greater activity because more of it reaches the cancer site in vivo and is more uniformly distributed. Autoradiography studies are planned in which comparisons of DT2219ARL to its monomeric forms might tell us how labeled DT2219ARL is reaching its tumor target. For example, some antibodies have high spatial heterogeneity in tumors because they are higher affinity and more prone to bind the first wall of antigen (binding site barrier) that they encounter after diffusion from the blood into the parenchyma [40]. Others are more homogeneously distributed and permeate tumor tissue better. These studies would shed light

on the tumor penetrating characteristics of DT2219ARL and may tell us if further affinity enhancements are warranted.

The original heavy chain hot spot mutation of DT2219EA consisting of three amino acids Thr-His-Trp (THW) in place of Ser-Ser-Tyr (SSY) at positions 100, 100A, and 100B in the anti-CD22 sFv CDR3 region enhanced its affinity [10]. Although Ho et al reported that light chain mutation of this same anti-CD22 sFv resulted in enhanced affinity [25], this was not the case when we incorporated these same S30G and N31R light chain mutations in DT2219EB1. The tertiary structure of a bispecific single chain cytotoxin is undoubtedly more complex and perhaps alteration of the light chain has less of an impact than alteration of the heavy chain. Also, it is possible that certain mutations that are beneficial to a monospecific cytotoxin may not be beneficial in a bispecific conformation. Still, the fact that heavy chain mutation did improve the affinity of DT2219EA proves that mutation can be used as a valuable tool to enhance cytotoxin affinity and further alterations of the heavy chain of anti-CD22 and/or anti-CD19 may prove extremely beneficial.

Another interesting aspect of this study is that DT2219ARL was not reactive with non-human primate cells. Parental RFB4 monoclonal antibody was reactive with human PBMC. Also, studies by others showed that the PE-based immunotoxin, BL22, made with the sFv from anti-CD22 RFB4 was reactive with cynomolgus monkey cells [41]. Surprisingly, no binding of macaque cells was observed in our study. Several explanations are possible. Perhaps, combining both sFvs together causes the recognition of a unique receptor not recognized by monomeric forms. Still, both monoclonals were able to block the activity of DT2219ARL in the proliferation assays. Our studies showed that HD37, the anti-CD19 parental monoclonal antibody was not reactive with macaque cells. Perhaps, the failure of macaque cells to bind anti-human CD19 somehow affected the overall binding ability of DT2219ARL. Regardless, the data indicate that macaque cannot be used as an “ontarget” model to study the preclinical toxicity of DT2219ARL and the FDA has recently approved an investigational drug IND for studying DT2219ARL in leukemia patients.

Our observation that a bispecific fusion toxin is superior is not unique to DT2219ARL and hematopoietic cancer, but has been observed for other bispecific fusion toxins which target solid tumors. For example, a bispecific IT assembled with sFvs simultaneously targeting EpCAM resulted in a bispecific IT that had significantly greater activity against the two monomeric IT in a colorectal cancer model [13]. Bispecific fusion toxin construction has not been limited to sFv ligands. Cytokines and interleukins can also be employed as ligands since studies show that EGF and IL-13 can be combined with DT390 on the same molecule and that this bispecific cytotoxin DTEGF13 shows superior ability to inhibit tumor growth compared to the monospecific forms [14–16]. Not all bispecific IT show superior activity than their monomeric counterparts (17).

Immunogenicity may or may not be a problem of DT2219ARL. In a recent study of a different anti-B cell immunotoxin, BL22 investigators found that BL22 was not immunogenic in patients with B cell CLL and most Hairy Cell leukemia patients receiving multiple cycles of treatment [40]. Perhaps, the more efficient the anti-B cell drug, the better the drug will be at inhibiting the B cell response (which includes generation of neutralizing antibody).

In summary, genetic engineering has resulted in an improved variant called DT2219ARL which not only has the improved ability to kill malignant B cells better than its monospecific counterparts, but it has the ability to produce long-term cancer survivors in two highly aggressive systemic human B cell malignancy models in scid mice. A genetically altered tumor line, Raji-luc, transfected with a bicistronic vector proved useful for following metastatic tumor progression and identifying tumor target organs. The superior activity of DT2219ARL was

dependent on the presence of 2 different ligands on the same single chain bispecific molecule. This agent could prove useful in an upcoming FDA IND approved phase 1 study as an alternative therapy for different chemotherapy resistant B cell malignancies including B-ALL, B-CLL, and B cell lymphoma.

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Abbreviations

ALL	acute lymphoblastic leukemia
ALT	alanine transfersase assay
BUN	Blood-Urea- Nitrogen Assay
DT2219	DT390 fused to two sFv, anti-CD22 and anti-CD19
DMEM	Dulbecco's Modified Eagle's Medium
DT	diphtheria toxin
DTCD22	DT390 fused to single anti-CD22 sFv
DTEpCam23	DT390 fused to two control sFv, anti-ErbB2 sFv and anti-EpCam
IC50	concentration at which 50% response is inhibited
FITC	fluorescein isothiocyanate
HLP	hind limb paralysis
IT	immunotoxin
Bic	DT390 fused to two anti-CD3 sFv
PBMC	human peripheral blood mononuclear cells
SLS	sodium N-lauroyl-sarcosine

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Figure 1.

A) Construction of DT2219ARL. 1) The original DT2219EA construct consisting of the first 389 amino acids of the DT (DT₃₉₀), the V_H and V_L regions of anti- CD22 (sFv) and anti-CD19 (31) linked by a 20 amino acid segment of human muscle aldolase (hma). 2) To construct DT2219ARL the V_H-V_L orientation was reversed and the V_L and V_H genes of each sFv were conjoined by a fragment encoding the ARL linker. 3) The final target gene was spliced into pET21d vector. B) SDS-PAGE gel containing all 3 DT2219 variants used in these studies. Lane 1 and 8 -Molecular weight standards, Lane 2-95 kDa DT2219ARL, Lane 3-DT2219EA, Lane 4-DT2219EB1, Lane 5-RFB4 monoclonal antibody, Lane 6-HD37 monoclonal antibody, Lane 7-DT₃₉₀ (partially purified), The gel was stained using Coomassie blue and shows size and purity of the agents.

Figure 2.

The in vitro effect of the DT2219 mutant proteins. A) Daudi cells were cultured with fusion proteins and proliferation was measured by uptake of tritiated thymidine. Data are percentage of control response where control response is untreated cells. Data are expressed as mean $\pm$ standard deviation (SD). The mean values of untreated Daudi cells were $121,001 \pm 8,276$ cpm/20,000 cells. B.) Selectivity was determined on the CD19⁺CD22⁺ HPBMLT cell line in a separate experiment. The mean cpm of untreated HPBMLT in this experiment was $61,993 \pm 7,178$ cpm/20,000 cells. DT2219ARL differed significantly from the control BIC3 group at 0.01 – 100 nM by Student t test ($p < 0.0001$). C) In a third experiment, the ability of the ligands themselves to mediate cytotoxicity was tested by inactivating the diphtheria toxin with the DT2219GE mutation that disrupts toxin activity and leaves the ligands intact. The mean cpm of untreated Daudi in this experiment was $112,164 \pm 10,379$ cpm/20,000 cells. D) The anti-proliferative effect of DT2219ARL, DT22, DT19, and a mixture of DT22/DT19 on Daudi cells was tested by measuring ³H-thymidine uptake 72 hours following IT exposure. Points on each graph represent mean of triplicate samples $\pm$ SD. Control counts = $61,993 \pm 7,178$ cpm/20,000 cells.

Figure 3.

The activity of mutated DT2219ARL is mediated by both anti-CD19 sFv and the anti-CD22 sFv ligands. Proliferation studies were performed in which Daudi cells were treated with a constant concentration of 10 nM DT2219ARL and then blocked with increasing concentrations of HD37 monoclonal antibody, RFB4 monoclonal antibody, or non-reactive control Ly5.2 antibody. Thymidine uptake was then measured. Each line represents the mean of triplicate determinations $\pm$ standard deviation (SD). Percent blocking was calculated in comparison to the unblocked control and then graphed. Counts for untreated Daudi cells were $59,301 \pm 2,804$ cpm/20,000 cells.

Figure 4.

Binding of DT2219ARL-FITC to monkey PBMC by direct immunofluorescence. Monkey PBMC, normal human PBMC, or normal human CD22+ magnetic bead enriched PBMC cells were incubated with DT2219ARL-FITC, or negative control DTEpCam23. Positive controls included FITC labeled conventional monoclonal antibodies RFB4-FITC or HD37-FITC. Flow cytometry was performed and data expressed as a contour plot showing cells versus increasing fluorescent intensity. The top 3 panels show human CD22+ magnetic bead enriched PBMC, the lowest 3 panels show normal monkey PBMC, while the middle 3 panels show normal human PBMC. The box in each panel outlines the gated area which shows binding that exceeds the values obtained for the negative control. The number in the box is the percentage of positive cells. NE- not evaluated.

Figure 5.

Groups of scid mice were given 10^6 Daudi cells iv to induce systemic disease. A) Three days following Daudi injection, mice were given the exact same injection schedule of multiple IP injections of DT2219ARL and DT2219EB1 in order to compare them to no treatment controls. Data was graphed as proportion surviving versus time. Statistical analysis was performed using the LogRank test and the DT2219ARL group significantly differed from the no treatment group ($p<0.001$). B) Three days following Daudi injection, mice were given ip treatment with DT2219EA and DT2219ARL in comparison to no treatment controls and to Bic3 immunotoxin control treated mice. The DT2219ARL group significantly differed from the DT2219EA group, the Bic3 group, and the no treatment group ($p<0.001$). C) Three days following Daudi injection, mice were given a single ip injection of DT2219EA and DT2219ARL in comparison to no treatment controls. Only the DT2219ARL group significantly differed from the no treatment group.

Figure 6.

Effect of IP administration of DT2219ARL on mice given systemic B cell cancer by IV injection of Raji-luc. Raji-luc cells stably expressing the luciferase gene were administered IV to scid mice. A) Mice were either treated with DT2219ARL (M1, M2, M3, and M4) on days 3, 5, 11, 16, and 18 or untreated (M5, M6, M7, and M8). Luciferase bioluminescence was measured as photons/sec/cm²/sr. B) The same data as shown in A is graphed in B. Data are expressed as total activity graphed over time for each individual animal (M1–M8). C) Digital Images of illustrating tumor progression in untreated Raji-luc mice. Bioluminescent imaging is shown for 3 untreated mice M9–M11 on day 21. Because the Raji-luc line has a GFP reporter gene as well as a luciferase reporter gene, fluorescent imaging is also shown for animals M9, M10, and M11. Lymphoma can be seen in lung, bone marrow, lymph node and compressing the spinal cord which likely causes hind limb paralysis (HLP). GFP imaging correlates with luciferase imaging.

Figure 7.

Toxicity of DT2219ARL in rabbits. Rabbits were given IV injection of DT2219ARL on days 1, 3, 5, 7. A) Average weight of two rabbits B) ALT enzyme levels from the same rabbits C) Frozen liver section from a rabbit treated with 500 ug/kg DT2219ARL. The section was stained with H and E and is shown at 100X magnification.

Table 1
K_d Values of DT2219ARL-FITC on various cells on malignant and normal B cells

	K _d (nM)	R ²
Normal B Cells		
Human PBMC	28	0.90
Enriched CD22 ⁺ Cells	20	0.95
Malignant B Cells (Cell lines)		
Daudi	133	0.97
Raji	39	0.99
Malignant B Cells (Patient B-CLL cells)		
Patient 1	36	0.97
Patient 2	181	0.99
Negative Control IT		
Binding to Raji		
DTe23EpCAM-FITC	1879	0.99

DT2219ARL was labeled with FITC using our standard labeling procedure and verified at 2–3 FITC molecules/DT2219ARL molecule. Cells were incubated with DT2219ARL-FITC in the dark, washed, and then run assayed on a Becton-Dickinson FACSCaliber. K_d values were determined using PRISM software. R² values indicate how well the regression plots fit data points.