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Safety evaluation of DT$_{388}$IL3, a diphtheria toxin/interleukin 3 fusion protein, in the cynomolgus monkey

Abstract We developed a fusion toxin, DT$_{388}$IL3, consisting of the catalytic and translocation domains of diphtheria toxin (DT$_{388}$) linked to interleukin 3 (IL3) for the treatment of patients with acute myeloid leukemia (AML). Our goal in this study was to estimate a range for the maximum tolerated dose (MTD) and to evaluate the dose-limiting toxicity (DLT) of DT$_{388}$IL3 in cynomolgus monkeys (*Macaca fasicularis*), which possess cross-reactive IL3 receptors. In our previous study, we administered up to six infusions of DT$_{388}$IL3 at 40, 60, or 100 μg/kg every other day to three pairs (one male monkey and one female monkey) of young adult monkeys. In five of six monkeys, results showed a dose-dependent increase in malaise and anorexia but no consistent abnormalities in serum chemistries or blood counts. There was no evidence of organ damage by blood tests or histopathology. However, the female treated at 100 μg/kg, died of moderate to severe vasculitis of multiple tissues. Based on these findings, this study repeated the 100 μg/kg group and added a group that received 150 μg/kg in an effort to confirm a dose response. Two female monkeys were treated with up to six infusions of DT$_{388}$IL3 at 100 μg/kg or 150 μg/kg every other day. One additional female monkey was treated as a negative control. Monkeys in the 100 μg/kg group showed moderate malaise and anorexia, but no consistent abnormalities in blood counts or serum chemistries. Moderate elevations of liver enzymes were noted in the 150 μg/kg group in addition to severe malaise and anorexia. No significant findings were revealed at gross necropsy. The histopathological findings revealed regenerative myeloid hyperplasia and hepatic degeneration and regeneration in the 150 μg/kg group. Similar lesions of less severity were detected in the 100 μg/kg group. DT$_{388}$IL3 plasma half-life was approximately 20 min with a peak concentration of approximately 2 μg/ml (30,000 pM). The IC$_{50}$ for AML blasts in vitro was 6 pM. Collectively, our results suggest that DT$_{388}$IL3 can be tolerated at doses up to 100 μg/kg in a nonhuman primate, which is higher than previously reported for other AML directed diphtheria toxin fusion proteins, and should in principle allow for dose escalation with reduced toxic side effects. Based on these findings a phase I clinical trial has recently been initiated with DT$_{388}$IL3 for the treatment of AML.

Keywords Diphtheria toxin · Cynomolgus monkey · Fusion protein · DTIL3 · AML

Introduction

Acute myeloid leukemia (AML) is the most common form of leukemia in adults and accounts for approximately 15–20% of childhood leukemia. The average incidence of AML typically ranges between two and six cases per 100,000 individuals in the USA [22]. Despite aggressive treatment, the prognosis remains poor as most patients that attain complete remission from induction combination chemotherapy relapse and die from chemoresistant disease and complications of therapy [23]. Development of selective cytotoxic agents that can circumvent the mechanisms of chemoresistance could greatly improve AML therapy.

One novel class of therapeutics is fusion proteins composed of catalytic toxins covalently linked to AML-selective peptide ligands. The ligand directs the molecule to the surface of the immature myeloid cell and triggers receptor-mediated endocytosis. The toxin then translocates to the cytosol and catalytically inactivates protein synthesis leading to cell death.

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Diphtheria toxin (DT) is a 535 amino acid residue protein with three domains [7]. The C-terminal domain (amino acid residues 390–535) has a β-sheet-rich tertiary structure and functions to bind the protein to the heparin-binding epidermal growth factor (EGF)-like cell surface receptor [6]. The middle domain (amino acid residues 201–389) is rich in amphipathic α-helices and facilitates translocation of the catalytic domain to the cytosol [24]. The N-terminal domain (amino acid residues 1–200) is an ADP-ribosylase and catalytically adds ADP to the diphthamide residue of elongation factor 2 (EF2) leading to inactivation of protein synthesis [5]. The C-terminal receptor-binding domain has been replaced with alternative ligands to generate fusion proteins [30].

Our initial attempt to target AML with fusion proteins was made using $DT_{388}GMCSF$ composed of the catalytic and translocation domains of DT (DT388) fused to human granulocyte-macrophage colony-stimulating factor (GM-CSF). In Phase I clinical trials, complete and partial remissions were seen; however, hepatic toxicity was dose limiting [13]. We recently demonstrated that $DT_{388}IL3$ targeting IL3 receptors was better tolerated in rodent models and lacked hepatic injury [32]. We engineered $DT_{388}IL3$ composed of DT (DT388) fused to human interleukin-3 (IL3) [12, 14, 18]. IL3 is a cytokine which supports the proliferation and terminal differentiation of multipotential and committed myeloid and lymphoid progenitors, but does not act on the most primitive hematopoietic stem cells [26]. Many human AML blasts express the IL-3 receptor and proliferate in response to IL3 [1]. In vitro studies of $DT_{388}IL3$ showed potent blast cell kill (greater than 1 log) from 36% of patients under conditions which produced minimal damage to normal hematopoietic stem cells [2, 3, 10, 12, 14]. In vivo studies using NOD/SCID mice inoculated intravenously with human IL-3 receptor positive AML blasts treated with $DT_{388}IL3$ daily for 5 days yielded a significantly improved median disease-free survival to >120 days ($P < 0.001$) [4, 17]. The maximum tolerated dose (MTD) of $DT_{388}IL3$ was 0.045 μg/g/day given as intra-peritoneal injections for 5 days [10]. In vivo studies using $DT_{388}IL3$ did not produce Kupffer cell or liver damage, which had been previously observed with $DT_{388}GMCSF$ [32].

In our previous study [9], we evaluated the toxicology and pharmacokinetics of $DT_{388}IL3$ in cynomolgus monkeys because they possess cross-reactive receptors to human IL3. We administered up to six infusions of $DT_{388}IL3$ at 40, 60, or 100 μg/kg every other day to three pairs of young adult cynomolgus macaques (one female and one male per pair). We reported the MTD of $DT_{388}IL3$ to be 60 μg/kg for six doses. Monkeys treated with 100 μg/kg $DT_{388}IL3$ exhibited severe malaise and anorexia with the female monkey dying of severe vasculitis of multiple organs. In this study, we attempt to estimate a more accurate range for the MTD and to add data regarding the dose-limiting toxicities.

Methods

Animals

Three young adult female cynomolgus monkeys weighing 2.5–3.5 kg were obtained from Charles River Company, Sierra Biomedical Division, Sparks, NV, USA and two were obtained from Worldwide Primates, Miami, FL, USA and quarantined for 60 days at the Comparative Medicine Clinical Research Center of Wake Forest Health Sciences. All procedures involving nonhuman primates were conducted in compliance with state and federal laws of the US Department of Health and Human Services and guidelines established by the Wake Forest University Institutional Animal Care and Use Committee. Monkeys were housed in single cages. Individual physical exams and baseline laboratory data to include complete blood count, biochemical panel, urinalysis, and clotting profile were recorded for each monkey.

Catherization and tethering system

Three weeks prior to catheter implantation, each monkey was acclimated to a nylon jacket (Alice King Chatham, Inc., Los Angeles, CA, USA) and tether for 3 weeks. After becoming adapted to the jacket tether system, each monkey had a silastic vascular catheter implanted into the femoral vein using sterile techniques. Surgery was performed under anesthesia using ketamine hydrochloride (Fort Dodge, Ft. Dodge, IA, USA), diazepam (Abbott Laboratories, North Chicago, IL, USA), and buprenorphine (Reckitt & Colman, Richmond, VA, USA) (15.0, 1.0, and 0.01 mg/kg, respectively). The catheter was inserted into the femoral vein and advanced into the caudal vena cava. The free end of the catheter was tunneled, subcutaneously, to exit the back between the scapulae and threaded through a flexible metal tether and attached to the swivel apparatus (Alice King Chatham) on the back of the cage. Normal saline with 1 U heparin/ml was infused at a constant rate of 3 ml/h through a three-way stopcock to maintain catheter patency.

Toxicology study

Two pairs of female monkeys were administered intravenous injections of 100 μg/kg or 150 μg/kg of $DT_{388}IL3$ every other day for a total of up to six doses. A fifth female monkey served as a negative control. The every other day regimen allows for observations of toxicities between doses and matches the planned therapeutic regimen in human patients. $DT_{388}IL3$ was diluted in 250 mM NaCl and 10 mM Tris HCL to a final volume of 1 ml for injection. All monkeys received prophylactic administration of vancomycin

hydrochloride, 10 mg/kg, (Abbott Labs) and ceftazidime, 30 mg/kg, (GlaxoSmithKline, England) intravenously throughout the length of the study. Infusion rate of heparinized saline was increased up to 6 ml/h as needed to maintain hydration. Animals were monitored daily for signs of clinical toxicity to include depression, lethargy, anorexia, diarrhea, vomiting, and pain. A toxicity grading system was adapted for monkeys as previously described [27]. Animals were treated with 3 mg/kg of ketoprofen, (Fort Dodge) or 0.1 mg/kg of buprenorphine, as needed for analgesia. Complete blood chemistries were performed daily and serum chemistries performed at least every 3 days. Chemistries included total protein, albumin, alanine transferase (ALT), alkaline phosphatase (ALKP), aspartate aminotransferase (AST), bilirubin, lactate dehydrogenase, blood urea nitrogen (BUN), creatinine, electrolytes, cholesterol, and triglycerides.

Pathology

Postmortem examinations were performed on all monkeys. Monkeys in the 100 μg/kg group (#7228, #7229) were euthanized on day 14. Monkeys in the 150 μg/kg were euthanized on day 6 (#7225) and day 16 (#7226). Samples from the adrenal glands, bone marrow, brain, catheter implantation site, cecum, cervix, colon, duodenum, eyes, heart, ileum, jejunum, kidney, liver, lungs, lymph nodes, mammary glands, nerve (sciatic), ovary, pituitary gland, prostate, skeletal muscle, skin, spleen, stomach, testis, thymus, thyroid gland, vagina, urinary bladder, and uterus were removed. The tissues were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. Sections were stained with hematoxylin and eosin and examined by a board certified veterinary pathologist (JMC).

Pharmacokinetics

On day 1 of $DT_{388}IL3$ infusion, 0.3 ml blood samples were collected via intravenous catheter at 0, 5, 30, 60, 90, 120, and 240 min. Samples were collected from the same catheter through which the experimental drug was administered. Serum samples were stored at −80°C until assayed. The concentration of $DT_{388}IL3$ in the blood was measured using a sensitive biological assay as reported previously [16]. Briefly, proliferation of TF1HRas leukemic cells in response to serial dilutions of serum was measured by tritiated thymidine incorporation. The serum concentration of the drug was derived from a standard curve using known concentrations of $DT_{388}IL3$. This assay yields reproducible values with a range of 30% with a limit of detection at 0.1 ng/ml. All assays were performed in triplicate. A nonlinear regression algorithim with GraphPad Prism was used to determine the $t_{1/2}$.

Detection of monkey IgG response to $DT_{388}IL3$

Pre-$DT_{388}IL3$-treatment and post-$DT_{388}IL3$-treatment blood samples (1.0 ml) were collected from the 150 μg/kg group and the serum separated and stored at −80°C until assayed. Serum anti-DT levels were detected by ELISA as previously described [16]. All samples were run in duplicate, and the mean values were calculated for analysis.

Results

Monkeys in the 100 μg/kg (#7228 and #7229) group received $DT_{388}IL3$ via intravenous injection every other day for six doses. In the 150 μg/kg dose group, monkey #7225 received $DT_{388}IL3$ via intravenous injection every other day for three doses (days 1, 3, and 5) and #7226 received five doses (days 1, 3, 5, 8 and 11).

Clinical observations and toxic changes for each monkey are summarized in Table 1. Monkeys in the 100 μg/kg group experienced little to no malaise or anorexia. Serum chemistry results revealed a transient elevation in bilirubin values in both monkeys and a mild elevation in ALT in monkey #7229 (Fig. 2). There was no serum biochemical or clinical evidence of renal disease. Hematological results revealed no significant change in hematocrit, neutrophil or lymphocyte counts from baseline (Fig. 1).

Table 1 Side effects in cynomolgus monkeys treated with $DT_{388}IL3$ intravenously for up to six every other day doses

Dose (μg/kg every other day IV×6)

	100	100	150	150	Control
Toxicity	7228	7229	7225[a]	7226[b]	7227
Hematology					
Leukopenia	0	0	0	0	0
Leukocytosis	1	0	3	2	1
Thrombocytopenia	0	0	1	2	0
Anemia	2	3	2	3	2
Gastrointestinal/appetite					
Anorexia	1	1	3	3	0
Weight loss	0	0	1	0	0
Diarrhea	0	0	1	1	0
Liver					
Alanine transferase elevation	0	1	2	2	0
Hypoalbuminemia	2	2	3	2	0
Renal/metabolic					
Elevated blood urea nitrogen	0	0	0	0	0
Elevated creatinine	0	0	0	0	0
Hypokalemia	0	0	1	1	0
Overall health					
Activity level	0	0	3	2	0
Discomfort/malaise	1	1	3	3	0

Uckun toxicity grading system with grades 0–3 (0 within normal limits, 1 mild, 2 moderate, 3 severe)
[a]Through three doses
[b]Through five doses
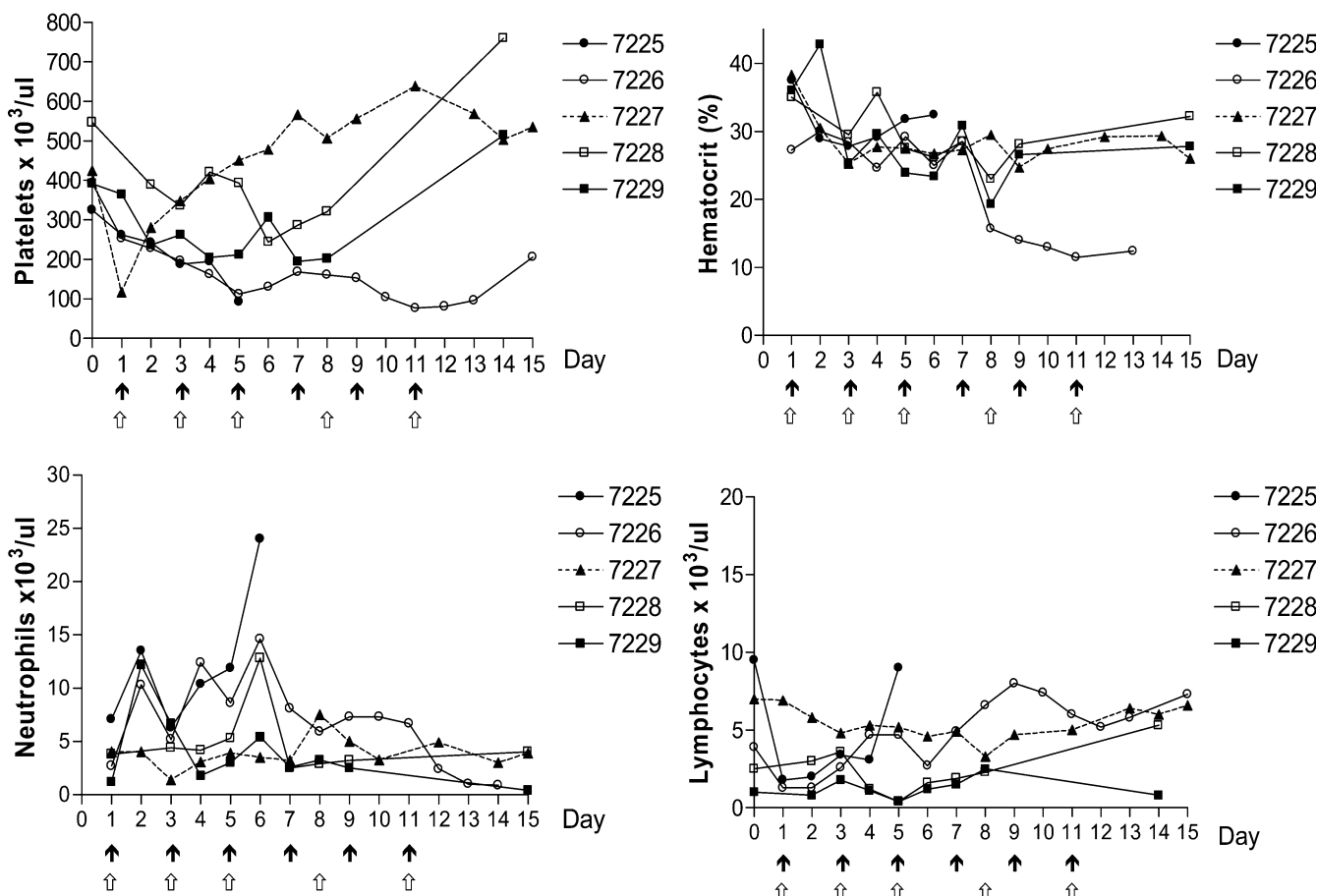
Fig. 1 Hematological results of monkeys treated with up to six intravenous infusions of $DT_{388}IL3$. *Black arrows* denote dosing schedule for the 100 µg/kg group; *white arrows* denote dosing schedule for the 150 µg/kg group

In contrast, both monkeys in the 150 µg/kg group (#7225 and #7226) presented with moderate to severe malaise and anorexia. Moderate elevations in ALT were observed in addition to mild to moderate hypoalbuminemia and transient hyperbilirubinemia in both monkeys (Fig. 2). However, there was no serum biochemical or clinical evidence of renal disease. Moderate to marked leukocytosis was observed in both female monkeys (Fig. 1). On day 6 of treatment, after receiving three $DT_{388}IL3$ infusions, Monkey #7225 presented moribund and was euthanized. A marked neutrophilia ($40.7 \times 10^3/ul$) with a left shift (16% bands), an elevated ALT value of 439 u/l, and a hypoalbuminemia of 2.7 g/dl was present at the time she was euthanized. Monkey #7226 had a similar, but less severe response and treatment intervals were extended to allow for recovery. Platelet counts in both dose groups and the negative control showed variability with the 150 µg/kg dose group showing mild to moderate thrombocytopenia (Fig. 2). Inadvertent blood loss due to catheter complications on days 4, 9, and 10 (monkey #7226) and days 2 and 9 (control monkey #7227) resulted in low hematocrit values (Fig. 1).

Necropsies were performed on all monkeys in both dose groups including the negative control. Monkeys #7228 and #7229 (100 µg/kg group) were euthanized 3 days after the sixth drug infusion on day 14. Monkey #7225 (150 µg/kg group) was euthanized 1 day after the third drug infusion on day 6 and #7726 (150 µg/kg group) 5 days after the fifth infusion on day 16. The control monkey (#7227) was euthanized on day 16. Grossly, no significant findings were observed in either dose group. Histopathological results for each monkey are shown in Table 2. Myeloid hyperplasia of mature cells was observed in treated monkeys except for #7225 of the 150 µg/kg group. In this animal, there was bone marrow necrosis with fibrin deposition and myeloid hyperplasia of immature cells. Evidence of vascular wall necrosis of venules of the lymph node was also observed. Additionally, within the kidney, there were mild, multifocal intraglomerular fibrin thrombi and tubular necrosis with regeneration. Evidence of renal pathology was not found in any other monkeys. Mild, diffuse lymphoid depletion of the spleen was noted in both 150 µg/kg treated monkeys. Extramedullary hematopoiesis was observed in the liver of the 100 µg/kg monkeys and in the adrenal glands of the 150 µg/kg monkeys.

The calculated half-life for $DT_{388}IL3$ was 20 min based on the results from the 150 µg/kg group (Fig. 3). $DT_{388}IL3$ concentration at 30 min post-intravenous

Fig. 2 Serum biochemical results of monkeys treated with up to six intravenous infusions of $DT_{388}IL3$. *Black arrows* denote dosing schedule for the 100 μg/kg group; *white arrows* denote schedule for the 150 μg/kg group
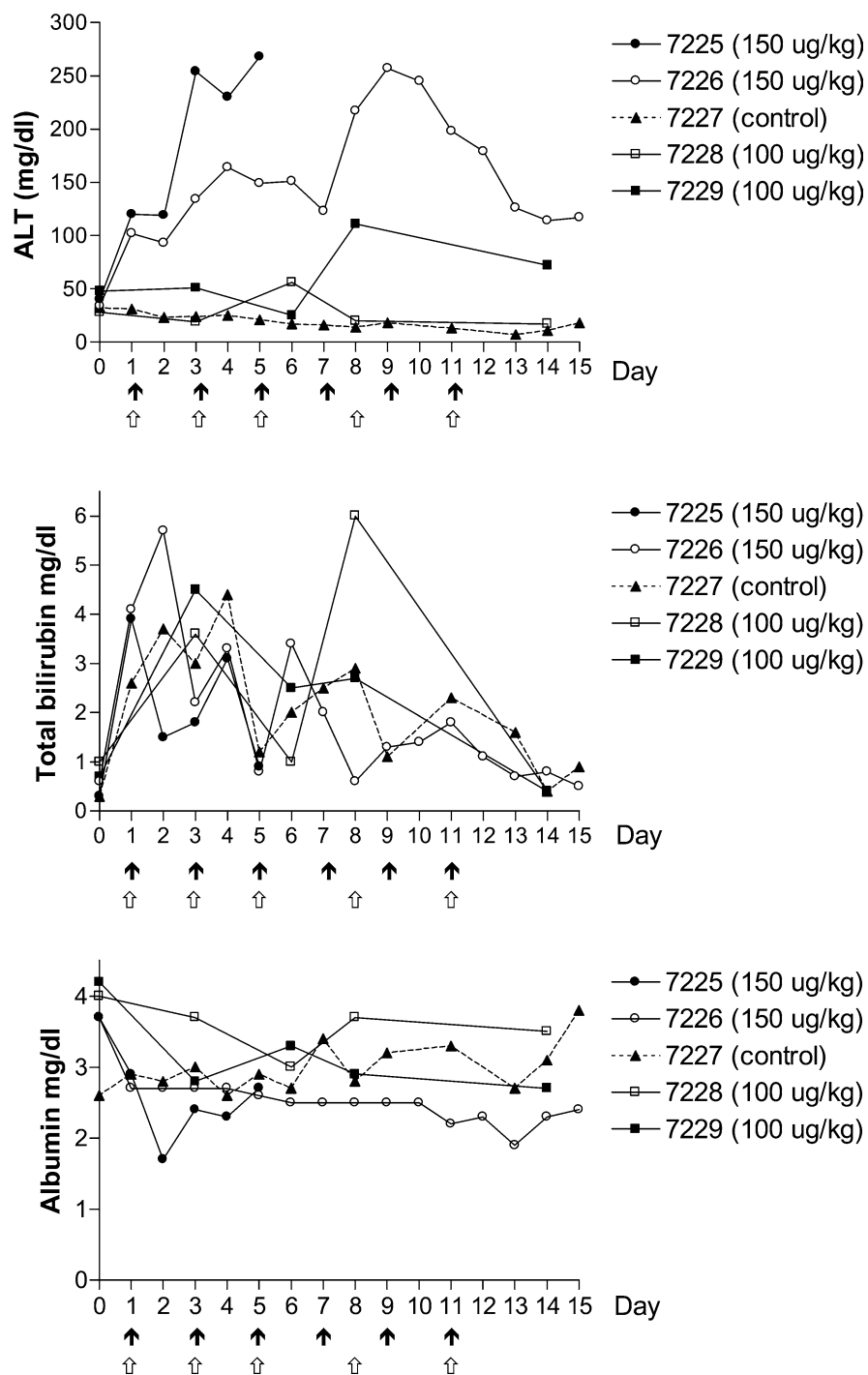
infusion was 3.9 μg/ml. Antibody responses were minimal in the 150 μg/kg group evaluated at 6 (#7225) or 16 (#7726) days post-treatment with anti-$DT_{388}IL3$ levels <1 μg/ml, see Table 3.

Discussion

In this study, we attempted to improve our knowledge regarding the safety and toxicity of the $DT_{388}IL3$ in nonhuman primates. Combining this study with our previous work, we observed tolerance of 100 μg/kg of $DT_{388}IL3$ in three of four animals. These data, while not statistically significant based on the small sample size, broaden the range of tolerated doses to include 100 μg/kg $DT_{388}IL3$. Previously tested fusion proteins in nonhuman primates have shown MTD's of 7.5 μg/kg/day for 5 days for $DT_{388}GMCSF$, 20 μg/kg/day for ten days for $DAB_{389}EGF$, 10 μg/kg/day for 14 days for $DAB_{389}IL2$, and 100 μg/kg for two doses for FN18-CRM9

Table 2 Histopathological findings of cynomolgus monkeys treated with DT$_{388}$IL3 intravenously for up to six every other day doses

Histopathological findings	100 ug/kg dose group		150 ug/kg dose group		
	#7228	#7229	#7225	#7226	#7227
Hepatocellular swelling	1	1	1	1	
Extramedullary hematopoeisis	1	1	1	2	
Myeloid hyperplasia	1	1	2	3	
Lymphoplasmacytic gastritis or colitis			1	1	1
Lymphoid depletion, spleen			1	1	
Lymphoid follicular hyperplasia	2	2			

1 mild, *2* moderate, *3* marked, *4* severe

Table 3 Antibody titers in cynomolgus monkeys treated with 150 µg/kg DT$_{388}$IL3 intravenously for up to five every other day doses

Monkey	Sample days	Anti-DT$_{388}$IL3 IgG (µg/ml)
7225	Day 0	< 0.02
	Day 6	< 0.02
7226	Day 0	< 0.02
	Day 16	0.5
7227[a]	Day 0	< 0.02
	Day 16	< 0.02

[a] Control monkey, not treated

[9]. Thus, only the anti T cell fusion protein has a similar nonhuman primate tolerance. In comparison, the MTD in humans for these fusion proteins is 4 µg/kg/day for DT$_{388}$GMCSF, 16 µg/kg/day for DAB$_{389}$EGF, and 27 µg/kg/day for DAB$_{389}$IL2 [20].

The dose-limiting toxicity (DLT) was vascular injury characterized by vascular leak syndrome (VLS) and fibrin deposition within blood vessels. This toxicity was dose related. In animals with mild to moderate vascular injury the removal of the drug resulted in reversal of the toxicity. Although recombinant human IL3 reacts with the macaque IL3 receptor and produces myeloid stimulation in vivo, the relative binding affinity of human IL3 for the monkey receptor had been reported to be 25-fold to 50-fold lower than for the homologous human receptor [28, 29]. Therefore, dose-limiting toxicities may not exactly parallel those seen in human patients.

Vascular leak syndrome is the dose-limiting side effect of many recombinant fusion toxin therapies including diphtheria, ricin and pseudomonas exotoxin fusion proteins [11, 25]. Vascular leak syndrome is characterized by an increase in vascular permeability accompanied by extravasation of fluids and proteins resulting in hypoalbuminemia, edema, weight gain, malaise,
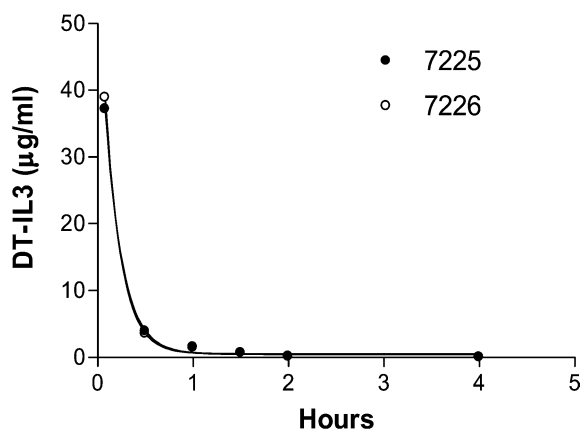
Fig. 3 Serum levels of DT$_{388}$IL3 for monkeys in the 150 µg/kg group. The half-life was determined to be approximately 20 min

anorexia, fatigue, and dyspnea. Both female monkeys treated at 150 µg/kg DT$_{388}$IL3 experienced clinical signs associated with VLS including moderate to severe hypoalbuminemia, malaise and anorexia similar to patients in phase II clinical trials of diphtheria fusion protein DAB$_{389}$IL2 [11]. Symptoms of VLS were temporarily alleviated by withdrawal of the fusion protein in our study. Additionally, both monkeys were maintained on constant rate infusion of intravenous saline to maintain hydration, which may have minimized hypotension. There is no known method to prevent VLS, however, the side effects are generally self-limiting. Attempts to reduce VLS have included modification of the immunotoxin molecule to increase specificity and thereby minimizing damage to vascular endothelial cells [25]. Construction of a small molecule inhibitor, endothelial cell (EC) myosin light-chain kinase (MLCK), which has been shown to protect the endothelium from injury from disease related stress, may also prove valuable in preventing VLS [31].

Administration of DT$_{388}$IL3 induced a mild to moderate rise in hepatic transaminases that subsided with cessation of the drug. These elevations correlate with the hepatocellular swelling and degeneration of hepatocytes observed histologically. Severity of the lesions was related to dose. This mild hepatotoxicity was transient and reversible as evidenced by decreasing ALT values and regenerative changes seen in hepatocytes histologically. Other fusion toxins have been shown to induce reversible liver damage including DAB$_{389}$IL2, ricin toxin A chain, and pseudomonas exotoxin. This is in comparison to diphtheria fusion toxins DAB$_{389}$EGF and DT$_{388}$GMCSF, which cause irreversible damage to hepatocytes and Kupffer cells, respectively [8; Marlena Moors Westcott, unpublished observations].

Myeloid hyperplasia of mature cells within the bone marrow and extramedullary hematopoiesis were observed in monkeys of both dose groups. This hyperplastic response is likely due to stimulation of IL3 receptors on multipotential and committed myeloid progenitors [26]. In contrast, monkey #7225 of the 150 µg/kg group had a myeloid hyperplasia of immature cells and necrosis within the bone marrow. Additionally, a circulating neutrophilia was also present in this animal. One explanation for this difference may be that IL3 receptors were present in abundance and located on

early and late progenitor cells [21, 26]. Alternatively, $DT_{388}IL3$ administration at such a high dose may have induced cytokine release resulting in a severe inflammatory response. Although bacterial sepsis could also yield similar findings, no evidence of infection was found. The variability in platelet counts observed between dose groups and the negative control could be due to a variety of factors including inflammation, inadvertent blood loss in some animals, or the presence of an indwelling silastic catheter.

The half-life of 20 min is similar to those of other DT fusion toxins. There were no pretreatment serum antibodies to $DT_{388}IL3$ and post-treatment antibody formation was low. Minimal exposure of up to 16 days to $DT_{388}IL3$ may explain the low antibody response. This response is similar to that seen in cynomolgus monkeys treated with $DT_{388}GMCSF$ and may reflect the low-dose administration of DT monomer in both studies [16]. In contrast, human patients that have been immunized with diphtheria toxoid during childhood may have sufficient titers to minimize efficacy of administered $DT_{388}IL3$ and should receive pretreatment screening [15].

In conclusion, this preclinical safety study suggests that an initial dose of $DT_{388}IL3$ may be identified that can be safely administered. In the phase I clinical trial of $DT_{388}IL3$, three patients received 4 μg/kg/day for six doses with minimal toxicity [19]. The results of this study should facilitate the design and testing of $DT_{388}IL3$ in current phase I clinical trials for the treatment of refractory AML.

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