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Safety toxicology of an IL-2 'no-alpha' mutein in the Sprague-Dawley rat following repeated dosing via intravenous administration

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ABSTRACT

The potential toxicity, safety and anti-drug antibody production of the novel IL-2 "no-alpha" mutein is in need of investigation as it may be a critical candidate for cancer therapy. The design of this mutein is meant to reduce toxicity compared to the IL-2 wildtype by disrupting interactions with the alpha receptor (CD25) and increasing the efficacy of the treatment. This was assessed following administration to Sprague-Dawley rats intravenously (IV), and it occurred over three cycles of five days each with daily dosing, with a 9-day washout period between each cycle. For the mutein dose groups, animals were dosed via IV at dose levels of 600, 6000, 18,000 U/kg. This dosing regimen is equivalent to 1x, 10x, and 30x the proposed first human dose, respectively. This study also assessed the progression or regression of any effects following a 14-day treatment-free recovery period for the control and high dose groups. Rats that were administered the "no-alpha" mutein at 600 and 6000 U/kg were well-tolerated with no apparent abnormal observations in general health, behaviour and autonomic function. There was no evidence of systemic toxicity based on evaluations in clinical pathology, gross necropsy and histopathology. At 18,000 U/kg (30x), abnormal clinical signs were observed at the injection sites consisting of localized swelling, discoloration, scabbing and necrosis. These animals showed a significant recovery in abnormal localized clinical signs following the treatment free period. Additionally other parameters did not indicate any significantly detrimental effects at this dose level. Therefore, the IL-2 mutein "no-alpha" seems to hold promise as a valuable addition to the current array of cancer therapy strategies, especially at the proposed dose level.

1. Introduction

Interleukin-2 (IL-2) is a pleiotropic cytokine, that is responsible for the generation, activation and mobilization of T lymphocytes and plays an essential role in the induction of a successful immune system [4,5]. It functions as both an autocrine and a paracrine growth factor for T cells and induces the proliferation of the natural killer cells (NK cells), increasing their cytotoxic activity. Specifically, IL-2 can stimulate the primary and secondary growth of T cells in addition to regulating subsets of CD4 + that are tasked with promoting and supporting cellular immunity against foreign bodies, giving its antitumor qualities [21].

This property led to its development as a viable cancer therapy that focused on melanomas and metastatic renal cell carcinomas (MRCCs), benefitting 15–30 % of patients [12,26,5]. However, this treatment required high doses which can cause life threatening toxicities mostly resulting from the Capillary/Vascular Leak Syndrome (CLS), limiting the use of this therapy in clinical practices [34,39,7]. This syndrome is partially mediated by the accumulation and direct signaling of the administered IL-2 on the endothelial cells of lungs and other organs, which happen to express CD25 (the alpha chain of IL-2R) and the high affinity trimeric IL-2 receptor [36]. Thus, CLS results in increased permeability of capillaries leading to plasma leakage and can potentially

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manifest in various detrimental conditions such as hypotension, prerenal azotemia, oliguria, pulmonary edemas, irregular heart contractions and even death [16,23,28,30].

Therefore, studies have investigated whether therapeutic benefits could be enhanced by creating novel muteins of IL-2, and one such mutein is named the "no-alpha" mutein. This mutein has shown higher levels of antitumor capabilities while lowering the level of toxic effects [11,4,6,5]. In experiments involving in-vivo treatment with mice that were administered the "no-alpha" mutein, the mutein selectively stimulated cytotoxic CD8 + T cells and NK cells, while insignificantly activating regulatory T cells [5]. The "no-alpha" mutein showed a larger anti- metastatic effect than other IL-2 variants in several transplantable tumor models [4,5].

Additionally, a clinical trial observing the "no-alpha" mutein in human patients with multiple localized tumors was also completed in the Phase I Clinical Assay under the title "Phase I/II trial with the mutein no alfa of IL-2: RPCEC00000234" ([10] - RPCEC00000234). Results from the trial suggested that treatment had reduced side effects and induced a large dose-dependent increase in absolute lymphocyte count ([10] - RPCEC00000234). The specific design of the "no-alpha" mutein was intended to reduce toxicity compared to IL-2 by disrupting interactions with the alpha receptor (CD25). This separation of IL-2's dual roles minimize its binding to regulatory T cells, which express CD25, and to the vasculature, thereby lowering the risk of toxic events like vascular leakage syndrome.

However, the safety toxicology aspects of the mutein have been little explored and is in much need of investigation prior to widespread use. It should be noted that IL-2 has a short in-vivo half life, therefore the study was based on previous animal toxicology studies conducted with a similar test item, Proleukin. The Proleukin has been administered to multiple animal models including monkeys, dogs and rats via IV dosing routes (Rosenberg et al., 1985; [22,8,1]). Based on these studies the dosing regimens for humans of Proleukin was determined to be 600,000 IU/kg of IV dosing every eight hours. This may continue up till 14 doses which is followed by a rest period and then followed by another cycle of dosing. The main difference between Proleukin and "no-alpha" mutein, is that the mutein selectively stimulates cytotoxic $CD8^{+}$T and NK cells, while reducing activation for regulatory T cells [5]. Given this dosing schema, and the similarities between Proleukin and the "no-alpha" mutein, the toxicological design for our current study was conducted in a similar manner.

The doses of the "no-alpha" mutein used in this study were chosen to highlight the balance between antitumor efficacy and manageable toxicity. These were based on IL-2 preclinical animal studies, Phase I dose-escalation trials as well as Phase II & III efficacy trials [3,27]. Phase II trials confirmed the efficacy of high doses of IL-2 in treatments of metastatic melanomas and renal cell carcinomas, providing adequate benchmarks for dose levels [17]. Dose levels were also influenced by comparative pharmacodynamics of IL-2 and the "no-alpha" mutein that were conducted in a preclinical setting [4], and preliminary results of the Phase I Clinical Assay ([10] - RPCEC00000234).

To build on previous assessments, in this current study, we investigated the safety of the "no-alpha" mutein in Sprague-Dawley rats by observing multiple comprehensive toxicological endpoints. This study did not incorporate model confirmation of markers or the effect on various immune cells after treatment administration as past preclinical studies had included this component ([10,4,6] - RPCEC00000234). These studies indicated that the "no-alpha" mutein increased proliferation of **NK** and CD8 + cells while not affecting the proliferation of regulatory T cells. Therefore, the focus of this study was to provide an accurate representation of the safety risks associated with this mutein. To our knowledge this is the first account of a multi-parameter investigation with various timepoints to investigate the safety of the IL-2 "no-alpha" mutein ".

2. Methods

This study was designed based on the guidelines for the safety testing of pharmaceutical products, particularly ICH (International Council of Harmonization) S6(R1) and S9 Guidelines, Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (ICH S6).

2.1. Materials

The mutein the "no-alpha" mutein was procured from the Department of Development of Process, Centre of Molecular Immunology, Havana, Cuba. The ethical approval number was 2021–59.AUP. Biological activity of this molecule was determined based on the colorimetric CTLL-2 cell proliferation assay developed by CIM. The control vehicle was composed of sodium acetate, EDTA disodium salt dihydrate, glycine and water for injection *quantum satis* (1 mL) and was prepared prior to each dose cycle.

2.2. Experimental animals

Sprague-Dawley rat strain CD®[Crl:CD®(SD)] was purchased from Charles River, Canada. Male and female rats were housed individually in Nalgene® rat cages. The animal room environment was controlled and monitored. LabDiet Certified Rodent Diet (#5002) and municipal water were offered ad libitum throughout the acclimatization and study periods. At the start of dosing, the age of the animals were 6–7 weeks. On average, the mean group body weight for males ranged from 166 – 250 g and for females ranged from 132 – 195 g at the start of dosing. The use of animals in this experimental protocol was approved by the Nucro-Technics Animal Care Committee.

2.3. Study outline

Following an acclimation period, rats were randomized into 4 groups (Group 1: control rats (vehicle); Groups 2, 3 and 4: rats were dosed with concentrations of low [600 U/kg], mid [6000 U/kg] and high [18000 U/kg] doses of the "no-alpha" mutein, respectively). The high dose group's dosing formulation was originally at 18,000 U/kg dosed in ~1 mL during dosing cycles 1 and 2, and then altered to 18,000 U/kg in ~2 mL to reduce irritation at injection sites. The total amount of protein administered was kept constant throughout the study period. The main study (ten animals/sex/group) and recovery animals (five animals/sex for groups 1 and 4) were dosed intravenously daily for three 5-day cycles with a 9-day washout period between each cycle, followed by a 14-day recovery period for the recovery groups (Table 1). Main study animals were used for the assessment of clinical pathology, gross necropsy and histopathology, while recovery animals were used to assess the reversal of toxicological effects over a two-week drug-free period. A subset of female rats dosed with the vehicle control and the high dose was used for capillary leak syndrome (CLS) investigation. The CLS investigation (five females each for groups 1 and 4) were dosed intravenously for five days during the first dose cycle followed by a necropsy approximately 1 hour after the last dose.

Table 1
Summary of the dosing cycle implemented in the study.

Dose Cycle	Dose Days	Washout Days
Cycle 1	Days 1–5	
Washout (8 days)		Days 6–13
Cycle 2	Days 14–18	
Washout (9 days)		Days 19–27
Cycle 3	Days 28–32	
Recovery Period (14 days)		Days 33–46

2.4. Safety assessment

The Sprague-Dawley rat was specifically selected due to the presence of a high-affinity receptor for Interleukin-2. The presence of this receptor was required to test the effects of IL-2 muteins at stimulating the antitumor activity of cytotoxic CD8 + T-cells and NK cells while not activating regulatory T-cells in the process. The intravenous route of exposure was selected because this is one of the intended routes of human exposure.

Three dose levels of the "no-alpha" mutein and a control vehicle group were administered by slow IV bolus injection over an approximate 2-minute period. The lateral tail vein was injected with a 26 G needle attached to a syringe daily for three 5-day cycles and a 9-day washout period between each dose cycle. This was followed by a 14-day recovery period at the end of Dose Cycle 3. During the dosing process rats were restrained in a rat restrainer. The volume of the administered test or control item was calculated based on the subject's most recently scheduled body weight.

All animals were observed twice daily during the study. Observations and measurements included general health, behaviour and autonomic function. The body weight of each rat was recorded weekly during acclimation period and weekly before dosing during the study and recovery periods. Food consumption was also recorded weekly during the study and recovery periods. Funduscopic (indirect ophthalmoscopy) and biomicroscopic (slip lamp) examinations were performed prior to treatment and during the last week of treatment.

Clinical pathology investigations (hematology, hemostasis, clinical chemistry and urinalysis) were performed prior to necropsy for all main study and recovery animals (Table 2). Regulations recommended by the Society of Toxicological Pathology for standard hematology, coagulation, serum and urine chemistry biomarkers were considered when collecting/analyzing samples [38]. Hematology parameters were determined using ADVIA 120 hematology analyzer system (Siemens Diagnostics). Coagulation parameters were analyzed by a mechanical coagulometric method using a STA Compact Analyzer (Stago Diagnostica). Clinical chemistry parameters were analyzed utilizing a Vitros XT 3400 (Ortho Clinical Diagnostics).

Table 2
Clinical Chemistry parameters that were analyzed at the end of Main Study and Recovery.

Hematology	
Hematocrit	Platelet count
Hemoglobin	Red blood cell count
Mean corpuscular hemoglobin	Reticulocyte count
Mean corpuscular hemoglobin concentration	White blood cell count
Mean corpuscular volume*	White blood cell differential
Morphology of cells	
Coagulation	
Activated partial thromboplastin time	Prothrombin time
Fibrinogen	
Clinical Chemistry	
A/G ratio*	Creatinine
Albumin	Globulin*
Alanine aminotransferase	Glucose
Alkaline phosphatase	Phosphorus
Aspartate aminotransferase	Potassium
Bilirubin (total)	Sodium
Calcium	Total protein
Chloride	Triglyceride
Cholesterol	Urea nitrogen
Urinalysis	
Bilirubin	pH
Blood	Protein
Colour and appearance	Sediment microscopy
Glucose	Specific gravity
Ketone	Urobilinogen
Leukocyte	Volume
Nitrite	

At the end of the treatment and recovery period, animals in each treatment group were weighed and then anesthetized using isoflurane. Blood samples were collected from the abdominal aorta, after which the rats were exsanguinated and subjected to necropsy. Each animal underwent an external examination, including a review of all clinically recorded lesions, as well as a detailed internal examination.

Organs and tissues were dissected, trimmed free of fat and weighed (Table 3). Absolute and relative organ weights (relative terminal body weight and brain weight) were calculated. Tissues and organs were retained from all animals for histological analysis.

Specifically, all tissues from the control, and high-dose the "no-alpha" mutein (main study and recovery animals), were prepared for microscopic examination by embedding in paraffin wax, sectioning and staining with Hematoxylin & Eosin (H&E) (Table 4). Additionally, target tissues/organs from the low and mid-dose groups (spleen, stomach and bone marrow) were also prepared in a similar manner. In regard to injection sites, histopathology evaluations were conducted for inflammation and vascular thrombosis. Inflammation was characterized by edema in the subcuticular region, infiltrates of mononuclear cells, neutrophils, and macrophages, and fibrosis. Vascular thrombosis was characterized by partial or complete occlusion of one or more large blood vessels by fibrin, fibrosis, or fibrosis with capillarization. When ranking severity of findings, a four-grade system was used: minimal, mild, moderate and severe.

2.5. CLS investigation

Observations for capillary leak syndrome were only conducted on designated female rats after the first dose cycle for study efficacy. Rats were dosed with either control or high dose the "no-alpha" mutein for five days and necropsies were performed approximately one hour after the last dose (Cycle 1 end). The fluid levels in the thoracic cavities were evaluated by measuring the volume of fluid (if present) using a syringe. Lungs were weighed and histopathology on lungs and kidneys (stained with eosin/hematoxylin) was performed. No other organ weights were taken and no other tissues were preserved for the animals in the CLS condition.

2.6. Anti-drug antibodies

Approximately two milliliters of blood was collected in plain vacutainers from all main study and recovery animals. This was conducted prior to necropsy. Following blood collection for the anti-drug antibody assessment, the blood was allowed to clot at room temperature for approximately 60 minutes. Afterwards, it was placed in a centrifuge for 20 minutes at 1300 RCF in order to separate the serum. The collected serum was stored at −10°C to −25°C. The testing for anti-drug antibodies (ADA) was conducted at Nucro-Technics using a validated method. This method used a sandwich enzyme immunoassay technique included a custom-made kit for ADAs to Mutein IL-2 by Somru BioScience ADA ELISA kit catalog SB-046–219 [Somru BioScience]. The "no-alpha" mutein was coated onto a 96-well microplate and relevant samples of Quality Control samples and test samples were pipetted accordingly. Anti- the "no-alpha" mutein antibodies that were present in the biological matrices were held in place by the immobilized the "no-alpha" mutein. Afterwards, the plate was washed, and any unbound

Table 3
Organs that were weighed at the terminal time points.

Adrenals	Pituitary gland
Brain	Prostate
Heart	Spleen
Kidneys	Testes
Liver	Thymus
Lung	Thyroid / parathyroids (weighed fixed)
Ovaries	Uterus

Table 4
List of tissues collected at terminal timepoints for control and high dose animals.

Abnormal Tissues	Injection Site	Sciatic Nerve
Adrenals	Jejunum	Skeletal Muscle
Animal Identification	Kidneys	Skin (inguinal) and subcutis
Aorta	Liver (sample of central & left lobes)	Spinal Cord (cervical)
Brain (3 levels)	Lungs (left & right)	Spleen
Cecum	Lymph Node (Mandibular)	Sternum & Marrow
Cervix	Lymph Node (Mesenteric)	Stomach
Colon	Lymph Node (Axillary)	Testes
Duodenum	Mammary gland	Thymus
Epididymides	Optic Nerves	Thyroid/Parathyroids
Esophagus	Ovaries	Tongue
Eyes	Pancreas	Trachea
Femur	Pituitary Gland	Urinary Bladder
Heart	Prostate	Uterus
Ileum	Salivary Gland (Submandibular)	Vagina

components were removed. An enzyme-linked protein is added and washed subsequently to remove any unbound proteins. A substrate solution was then added to the wells to detect concentration via color change. The color development was proportional to the amount of therapeutic reagent present within the observed samples. ADA analysis was performed for all study groups. The ADA analysis was a qualitative test only (cut-point screening assay).

2.7. Data analysis and statistics

In-life data was collected using the ToxData System v.3.0.6 (PDS Inc., U.S.A.). Necropsy data and organ weights were entered into the current PDSPath Data System V.10.1. The data for males and females were separately analyzed for homogeneity of variance and for normality. Homogeneous data were analyzed using the Analysis of Variance (ANOVA; $p < 0.05$) and the significance of inter-group differences was analyzed using Duncan's *t*-test. Heterogeneous data were analyzed using the Kruskal-Wallis test and the significance of inter-group differences between the control and treated groups was assessed using Dunn's *t*-test. Statistical results were interpreted in the light of all other available data, including historical data, clinical observations, clinical and morphologic pathology data, and all known biological variables for rats, including known incidental and background data.

3. Results

3.1. Safety Assessment

3.1.1. Clinical observations

All subjects survived to the scheduled necropsy dates. Daily clinical observations (cage side monitoring and handling of animals) and detailed weekly physical observations showed localized test-article related findings in high dose animals (Table 5). All animals in the high dose (18,000 U/kg) group exhibited either one or more signs of discoloration, swelling, eschar and scabbing of the tail during the second cycle of administration. These signs were consistent with necrosis of tail tissue and as a result dosing was suspended for 1–2 days in the second cycle for select animals with pronounced abnormal clinical signs. This was conducted for nine males and six females of the high dose group. These adverse signs lessened during the second washout period and dosing safely resumed in the third cycle with modifications to the dosing regimen. In the third cycle the high dose group received the same total dose but used a lower delivery concentration with a higher volume to reduce the localized aggravation from administration. With this modification all high dose animals successfully completed treatment in the third cycle, however they still displayed injection site lesions, swelling, discoloration, and partial necrosis. It should be noted that the partial necrosis was less severe in comparison to the second cycle of dosing. These abnormal injection site findings improved over the course of the recovery period and the clinical sign of swelling became nonexistent in high dose animals by the second day of the recovery period.

Table 5
Summary of abnormal tail observations in high dose main study animals by cycle, as control, low and mid dose animals rarely showed abnormalities.

Sex	Sign	Cycle 1 (N = 15) Days 1–13	Cycle 2 (N = 15) Days 14–27	Cycle 3 (N = 15) Days 28–35	Recovery (N = 5) Days 36–47
Male	Discoloration	0	10	8	3
	Swelling	0	15	15	3*
	Scabbing	0	5	5	1
	Eschar	0	1	1	0
Female	Discoloration	0	7	9	3
	Swelling	0	15	15	5*
	Scabbing	0	1	1	1
	Eschar	0	0	0	0

* Indicates that abnormal sign was resolved in all recovery animals by Day 37.

All animals in the control, low and mid dose groups within the main study did not show any abnormal signs during the study period. Similarly, the control group within the recovery study also appeared normal during the study.

3.1.2. Body weights and food consumption

Food consumption, body weights and body weight changes were similar across all treatment groups within the main study. Food consumption was also not affected by the treatments when in the recovery phase. However, in one instance during the recovery period (Day 40), the mean body weights of high-dose males (m=368.2 ± 25.4 g) were significantly lower than control (m= 431.8 ± 39.3 g; $p < 0.05$). However, in the week prior to and post this observation, the weights between these two groups did not show a significant difference. Additionally food consumption was not affected, thus this observation was deemed not test item related. At the end of the recovery period, there were also no significant differences to be reported, further supporting that it is likely incidental.

3.1.3. Ophthalmological findings

At the end of the treatment period, some animals displayed new ophthalmological findings that were absent during the pre-study period. Specifically, there were four in high dose, three in mid dose, two in low dose and one in the control group that displayed subepithelial crystalline deposits. One male rat in the low dose group also displayed a punctate cataract. Lastly, one female rat in the high dose group displayed an anterior uveitis and hyphemia.

3.2. Anti-drug antibodies

Rat serum samples were analyzed for anti-drug antibodies using a validated ELISA method for the determination of antibodies to the "no-alpha" mutein. A summary of positives per group can be seen in Fig. 1. At the end of the main study period, sex combined data showed that 0 % of control animals, 65 % of low-dose, 60 % of mid-dose, and 95 % of high-dose animals tested positive for antibodies against the "no-alpha" mutein. At the end of the recovery period, sex combined data indicated that 0 % of control and 100 % of high dose animals tested positive.

3.3. CLS Study

No test-item-related microscopic findings were noted in the kidneys or lungs of the capillary leak evaluation subgroup after a single 5-day dose cycle. In addition, no test-item-related significant differences were observed for body weight or lung weight in high-dose animals compared to controls.

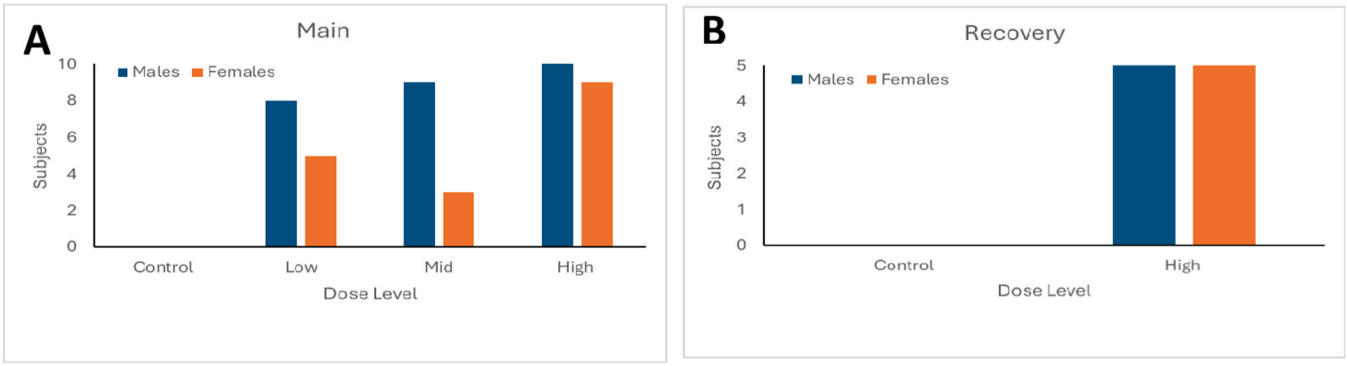

Fig. 1. A – Represents the presence of anti-drug antibodies found in the Main study period; n = 10 per treatment. B – Represents the presence of anti-drug antibodies found in the Recovery period; n = 5 per treatment. Control animals did not show any indication of anti-drug antibodies.

3.4. Clinical pathology

This section included multiple parameters at various timepoints. To summarize key findings, only statistically significant differences will be outlined in tables. Parameters that are not included in tables were excluded, because in those parameters all treatment groups exhibited values that were statistically similar to control animals.

3.4.1. Hematology

At the end of the treatment period, statistically significant increases in a few hematology parameters were observed and are reported in Tables 4 and 5, however most are within normal ranges except for neutrophil count. On Day 35, males in the high dose group had 4.7 % and 4.6 % lower hemoglobin ($p < 0.05$) and hematocrit ($p < 0.01$) respectively compared to control. High dose males also had 72.2 % increase in neutrophil count, compared to control ($p < 0.05$). Females in the high dose group had 6 % lower red blood cell count ($p < 0.05$), and 117.7 % higher neutrophil count compared to control ($p < 0.01$). High dose females also had a 78.3 % 91.3 % increase in reticulocyte concentration and proportion, compared to control ($p < 0.01$). At the end of the recovery period, high dose males displayed a 24.6 % increase in reticulocyte count, compared to control ($p < 0.05$). Additionally at the end of recovery, high dose females displayed a 11.3 %, 9.9 % and 10.9 % decrease in hemoglobin, MCH and MCHC respectively, compared to control ($p < 0.05$).(Tables 6 and 7)

3.4.2. Clinical chemistry

All treatment groups presented similar findings for the various clinical chemistry parameters, when compared to control at the end of the main study period. However, at the end of the recovery period, there were two parameters that were significantly different than control (Table 8). High dose females displayed a 28.1 % increase in glucose ($p < 0.05$), and high dose males displayed 7.7 % decrease in the albumin/globulin ratio compared to control ($p < 0.05$). It should be noted that in both instances, values were within the normal ranges for the relevant parameters.

3.4.3. Urine analysis and coagulation

Urine analysis indicated that all treatment groups were statistically similar to control among the various parameters, both at the end of the main study period and recovery. Coagulation parameters exhibited comparable trends, in which treatment groups were not statistically different from control at the end of both periods.

3.5. Gross pathology

After the gross necropsy procedure, findings indicated that the mutein's effects were primarily localized at the injection site and affected only the high dose group. Throughout the main study, necrosis (black discoloration), scabs, and scars were observed in 8/10 high-dose male subjects and 6/10 high-dose female subjects. In the recovery study period, these findings were observed in 4/5 high-dose male subjects and 3/5 high-dose female subjects.

3.6. Organ weights

Statistically significant differences from the control group were found for mean organ weights (a), organ to body weight percent ratios (b), and organ to brain weight percent ratios (c). These findings are reported in Table 9. Mid dose males displayed spleen weights that were 18.9 % less for (a) ($p < 0.05$), 19 % less for (b) ($p < 0.01$) and 20.3 % less for (c) when compared to control ($p < 0.01$). Mid dose females displayed lung weights that were 20.2 % more for (c) when compared to control ($p < 0.05$). Mid dose females displayed thyroid/parathyroid weights that were 21.1 % more for (a) ($p < 0.05$), 18.8 % more for (b) ($p < 0.05$) and 35 % more for (c) when compared to control ($p < 0.01$).

Table 6
Male Hematology Parameters – Mean values and percent changes from control group, highlighting statistical significance for neutrophils, hemoglobin, hematocrit and reticulocytes.

Timepoint	Parameter	Control	Low Dose	Mid Dose	High Dose	Normal Range
End of Treatment	*NEU [x10^9/L]*	0.90	1.00 (+11.1 %)	0.92 (+2.2 %)	1.55* (+72.2 %)	**0.47–1.51**
	HEMO [g/L]	148	149 (+0.7 %)	147 (−0.7 %)	141** (−4.7 %)	139–162
	HEMA [%]	45.5	45.7 (+0.4 %)	45.0 (−1.1 %)	43.4* (−4.6 %)	41.8–49.1
Recovery	*RET [x10^9/L]*	192.4	-	-	239.8* (+24.6 %)	138.8–239.8

"NEU" – Neutrophil count, "HEMO" – Hemoglobin, "HEMA%" - Percent of Hematocrit, "RET" – Reticulocyte count.
* Statistically different from control ($p < 0.05$),
** Statistically different from control ($p < 0.01$), bolded value indicates that a group was out of range based on historical data from our colony records.

Table 7
Female hematology parameters – Mean values and percent changes from control group, highlighting statistical significance for neutrophils, red blood cells, reticulocytes, hemoglobin, mean corpuscular hemoglobin.

Timepoint	Parameter	Control	Low Dose	Mid Dose	High Dose	Normal Range
End of Treatment	*NEU [x10^9/L]*	0.62	0.47 (−24.2 %)	0.69 (+11.3 %)	1.35** (+117.7 %)	**0.27–0.96**
	RBC [x10^{12}/L]	7.51	7.44 (−0.9 %)	7.60 (+1.2 %)	7.06* (−6.0 %)	6.84–8.36
	RET [x10^9/L]	138.5	157.3 (+13.6 %)	163.3 (17.9 %)	246.9** (+78.3 %)	88.1–265.2
	RET [%]	1.84	2.12 (+15.2 %)	2.16 (+17.4 %)	3.52** (+91.3 %)	1.12–3.54
Recovery	*HEMO [g/L]*	159	-	-	141* (−11.3 %)	135–155
	MCH [pg]	21.2	-	-	19.1* (−9.9 %)	17.3–19.2
	MCHC [g/L]	376	-	-	335* (−10.9 %)	323–354

"NEU" – Neutrophil count, "EOS" – Eosinophil count, "EOS %" - Percent of Eosinophil, "RBC" – Red Blood Cells, "RET" – Reticulocyte count, "RET%" - Percent of Reticulocytes, "HEMO" – Hemoglobin, "MCH" – Mean Corpuscular Hemoglobin, "MCHC" - Mean Corpuscular Hemoglobin Concentration.
* Statistically different from control ($p < 0.05$),
** Statistically different from control ($p < 0.01$), bolded value indicates that a group was out of range.

Table 8
Clinical Chemistry Parameters – Mean values and percent changes from control group, highlighting statistical significance in glucose and A/G ratio levels.

Timepoint	Parameter	Control	Low Dose	Mid Dose	High Dose	Normal Range
Recovery (female)	*GLU [mmol/L]*	6.4	-	-	8.2* (+28.1 %)	6.5–13
Recovery (male)	*A/G (ratio)*	1.3			1.2* (−7.7)	1–1.3

"GLU"- Glucose, "A/G" – Ratio of Albumin/Globulin.
* Statistically different from control ($p < 0.05$)

Table 9
Statistically significant values in organ weight comparisons – main study period. Splee, thyroid/parathyroid and lungs show statistical differences when compared to control ($p < 0.05$, $p < 0.01$).

Organ		Males			
		Control	Low Dose	Mid Dose	High Dose
spleen	*mean weight*	0.6890 g	0.6228 g (−9.6 %)	0.5588 g* (−18.9 %)	0.6520 g (−5.4 %)
	organ/body (%)	0.1882	0.1695 (−9.9 %)	0.1524** (−19.0 %)	0.1840 (−2.2 %)
	organ/brain (%)	35.2110	31.2144 (−11.4 %)	28.0719** (−20.3 %)	32.9289 (−6.5 %)
thyroid and parathyroid	*mean weight*	24.343 mg	25.583 mg (+5.1 %)	21.875 mg (−10.1 %)	21.726 mg (−10.8 %)
	organ/body (%)	0.007	0.006 (−14.3 %)	0.006 (−14.3 %)	0.006 (−14.3 %)
	organ/brain (%)	1.243	1.186 (−4.6 %)	1.095 (−11.9 %)	1.091 (−12.2 %)
Organ		**Females**			
		Control	**Low Dose**	**Mid Dose**	**High Dose**
lungs	*mean weight*	1.0876 g	1.0989 g (+1.0 %)	1.1762 g (+8.2 %)	1.2000 g (+10.3 %)
	organ/body (%)	0.5189	0.5014 (−3.4 %)	0.5507 (+6.1 %)	0.5548 (+6.9 %)
	organ/brain (%)	57.7972	60.2322 (+4.2 %)	69.4438* (+20.2 %)	66.3729 (+14.8 %)
thyroid and parathyroid	*mean weight*	16.531 mg	19.105 mg (+15.6 %)	20.016 mg* (+21.1 %)	17.112 mg (+3.5 %)
	organ/body (%)	0.008	0.009 (+12.5 %)	0.009* (+18.8 %)	0.008 (+0.9 %)
	organ/brain (%)	0.881	1.051 (+19.2 %)	1.189** (+35.0 %)	0.945 (+7.3 %)

* Statistically significant from control ($p < 0.05$), ** Statistically significant from control ($p < 0.01$)

3.7. Histopathology

Test item related histopathological findings in the main study were noted at the injection sites, tail, and spleen of the high dose rats. Injection site findings included minimal to moderate hemorrhages, minimal to moderate inflammation, minimal to moderate vascular

thrombosis, that in some animals were associated with tail necrosis. The frequency and severity of findings at the middle and tip injection site for main study animals are reported in Tables 10 and 11. The prevalence and severity of each of these findings were greatest in the high dose group. Additionally, there was an increase in lymphocytes in the peri-arterial lymphatic sheath of the spleen for animals given the test item.

Additionally, abnormal histopathological findings related to the mutein were observed at the injection sites and tails of the high dose animals in the recovery stage. The injection site observations included minimal to mild hemorrhages in the high dose males, as well as minimal to mild inflammation and minimal to moderate vascular thrombosis in high dose male and female groups. The tails displayed mild to severe inflammation, minimal to mild hemorrhages, minimal to severe vascular thrombosis, and mild to severe necrosis. The frequency and severity of these findings in recovery animals can be found below in Table 12 and Table 13.

4. Discussion

This study evaluated the overall preclinical safety of the IL-2 mutein known as "no-alpha" following intravenous administration in the Sprague-Dawley rat. The safety evaluation of the "no-alpha" mutein was based on an escalating dose toxicological assessment in the dose range of 600–18,000 U/kg/day. The safety assessment included monitoring of clinical signs, weekly detailed clinical observations, body weight, food consumption, clinical pathology, determination of anti-drug antibodies, gross pathology evaluation and histopathology examination. The study also evaluated the possibility of CLS following a 5-day treatment period with the "no-alpha" mutein since the wild type IL-2 high doses are known to cause CLS. For the purpose of the discussion, the treatment groups will be referred to as 1x group (low dose), 10x group (mid dose) and 30x (high dose). This is to highlight the magnitude disparity between observed groups and the proposed doses for humans.

4.1. Clinical signs

On a comparative basis, all doses of the "no-alpha" mutein were well tolerated, and rats in the control group, 1x group, and 10x group did not show any abnormal clinical signs. The only test item-related clinical observations were observed in rats given a dose 30 times higher than the human-equivalent dose. In this group, subjects of both sexes showed localized adverse effects like necrosis at the tail vein injection sites due to the high concentration of the "no-alpha" mutein in the dose formulation, especially after the first dose cycle. The abnormal tail findings had lessened during the 9-day washout period, but the dosing regimen for high dose animals was modified after cycle 2. Once the delivery concentration of the "no-alpha" mutein was lowered and the volume was increased to maintain the original dose level, the localized injection site findings diminished but were still present in the third dose cycle. Localized effects likely resulted from IL-2 product irritation and were not sex-specific. HDIL-2 is known to irritate the skin with the presence of rashes and can cause skin inflammation [35]. In a study with human patients in HDIL-2 therapy, 72 % of them displayed erythema and a few displayed necrotic lesions [40]. Our findings showed similar results; however, they were only observed when dosing with 30x the proposed dose. The fact that tail lesions showed signs of recovery during the washout period indicates that the effects are temporary and can be eliminated after discontinuing treatment. This was similarly seen in a human trial, in which cutaneous lesions receded after treatment was stopped [23]. Additionally, in human use, these localized effects are low-risk as they can be mitigated by better dosing routes in addition to the lowered proposed dose. In the Phase 1 human trials, a port-a-cath was used to administer the drug to great effect, in turn reducing the irritability of concentrated the "no-alpha" mutein ([10] - RPCEC00000234).

4.2. Body weights and food consumption

Evaluations of the body weights and food consumption data showed insignificant changes between groups and sexes. This indicates that regardless of dose level, appetite and natural weight gain were unaffected. This may be an advantage as some cancer therapies can reduce appetite causing anorexia, thus reducing effectiveness of the treatment [14].

4.3. Ophthalmology

Ophthalmological findings following dosing with the "no-alpha" mutein were viewed as unremarkable. While a higher presence of corneal deposits (crystallization) was observed post treatment, these are the most frequently diagnosed spontaneous occurring lesions in Sprague-Dawley rats [19]. Additionally, as the observed lesions were equally apparent throughout the groups with a lack of dose-dependency effects, these lesions were not considered to be treatment-related. The other two findings occurred in single animals as independent instances and were also not considered to be treatment related.

4.4. Erythrogram

Changes that were observed in the erythrogram such as HGB, HCT, RBC, and reticulocytes in the high dose group had little biological significance as the changes were minor and all values were within normal ranges. Reticulocyte presence was significantly increased in animals given 30x the proposed dose and this is most likely attributed to the localized signs from the trauma of dosing. Hemorrhages, thrombosis and inflammation were observed mainly in high dose groups and

Table 10
Summary of injection site (middle of the tail) findings in main study animals. High dose animals exhibited a higher severity of abnormal signs compared to other groups.

Finding		Males				Females			
		Control	Low	Mid	High	Control	Low	Mid	High
Hemorrhage	Grade 1	5	3	2	2	3	3	8	3
	Grade 2	0	1	3	7	1	0	0	3
	Grade 3	0	0	0	1	0	0	0	0
Inflammation	Grade 1	1	4	4	0	4	5	8	1
	Grade 2	0	0	2	9	0	0	2	8
	Grade 3	0	0	1	1	0	0	0	0
Thrombosis	Grade 1	0	1	4	2	0	1	5	1
	Grade 2	0	0	0	8	0	0	0	6

N = 10 for all groups; Grade criteria: 1 =Minimal; 2 =Mild; 3 =Moderate; 4 =Marked

Table 11
Summary of injection site (tail tip) findings in main study animals. High dose animals displayed a higher frequency of more severe abnormal signs compared to other groups.

Finding		Males				Females			
		Control	Low	Mid	High	Control	Low	Mid	High
Hemorrhage	Grade 1	1	0	0	1	0	0	0	1
	Grade 2	0	0	0	1	0	0	0	3
Inflammation	Grade 1	2	0	0	0	1	0	0	1
	Grade 2	0	0	0	2	0	0	0	5
	Grade 1	0	0	0	0	0	0	0	1
Thrombosis	Grade 2	0	0	0	2	0	0	0	4
	Grade 3	0	0	0	1	0	0	0	0
Necrosis	Grade 4	0	0	0	1	0	0	0	0
Degeneration	Grade 2	0	0	0	0	0	0	0	1

N = 10 for all groups; Grade criteria: 1 =Minimal; 2 =Mild; 3 =Moderate; 4 =Marked

Table 12
Summary of injection site (middle of the tail) findings in recovery study animals. High dose animals exhibited abnormal signs at a higher frequency and severity compared to control.

Finding		Males		Females	
		Control	High	Control	High
Hemorrhage	Grade 1	2	3	0	0
	Grade 2	0	1	0	0
Inflammation	Grade 1	0	1	0	3
	Grade 2	0	4	0	0
Thrombosis	Grade 1	0	1	0	2
	Grade 2	0	3	0	1
	Grade 3	0	1	0	0

N = 5 for all groups; Grade criteria: 1 =Minimal; 2 =Mild; 3 =Moderate

Table 13
Summary of injection site (tail tip) findings in recovery study animals. High dose animals displayed a higher frequency and severity of abnormal signs compared to control.

Finding		Males		Females	
		Control	High	Control	High
Inflammation	Grade 1	0	2	0	1
	Grade 2	0	0	0	1
Thrombosis	Grade 1	0	1	0	0
	Grade 2	0	1	0	1

N = 5 for all groups; Grade criteria: 1 =Minimal; 2 =Mild; 3 =Moderate

reticulocyte presence increases after trauma to blood vessels [24]. Small decreases in hemoglobin and hematocrit were also observed in the high dose groups and females continued to display a decrease after the recovery period. This may indicate that females are more susceptible to the mutein, however all values were within normal ranges. These signs indicate further that there was no sign of CLS, as CLS normally presents with increased levels of hematocrit and hemoglobin [9,33].

4.5. Leukogram

Changes in the leukogram included an increase in neutrophil count beyond normal ranges during the treatment period for the 30x dose level which returned to within normal ranges at the end of the recovery period. The change in neutrophil count likely reflects local tissue inflammation based on the injection site inflammatory response [35]. This is an expected result, as the effects of IL-2 muteins involve an increase of CD8 + T-Cell and NK Cell numbers, which have been shown to recruit and activate neutrophils [13,15,20]. When mice were dosed with IL-2 they found an increased presence of neutrophil and eosinophil infiltrates in the skin of the treated areas [25]. Additionally, a case study with a human patient after undergoing seven cycles of HDIL-2 therapy, showed histopathology results of necrotic keratinocytes in the skin layers and a presence of eosinophils [23]. These findings align with ours and the changes observed in the leukogram also returned to normal ranges at the end of the recovery period, indicating that effects are reversible after halting treatment. The increased immune function also explains the upregulation of glucose observed in high dose females as glucose is a required energy source for immune cells. High levels of glucose that are within normal ranges can expedite immune function and proliferation [32].

4.6. Gross pathology, histopathology, organ weights

Observations made for gross pathology, histopathology, and organ weights were either noted as unrelated to the IL-2 mutein dosing or were localized events resulting from the dosing route. For instance, significant differences in a few organ weights were found in animals dosed at 10x the proposed human dose but were not present in the 1x or 30x group, and therefore were deemed unrelated to the IL-2 mutein. This is similar to findings of Carmenate et al. [4] in which mice dosed with the "no-alpha" mutein showed no changes to liver and lung weight, while those dosed with the wild-type displayed significantly larger weights for both.

When assessing gross pathology and histopathology, most abnormal observations coincided with injection sites. This included hemorrhages, inflammation, thrombosis and necrosis. However, this is mainly attributed to the dosing route as mentioned previously when discussing the abnormal clinical signs. Therefore, issues arising from injection sites can be considered low-risk in human use as a more secure dosing routes can be implemented. As previously mentioned, a port-a-cath was used effectively in the Phase 1 clinical study and similar abnormal observations were reduced ([10] - RPCEC00000234).

Additionally, all animals that received the IL-2 mutein showed histopathological signs of increased lymphocytes within the periarterial lymphatic sheath of the spleen. At first glance it may seem like a symptom of CLS however it lacked any corroborating evidence. Therefore, this finding may be connected to the expected immunostimulatory effects of the mutein, which promotes expansion of CD8 + T cells and NK cells without inducing systemic inflammation or CLS [7]. The localized increase in lymphocytes may also reflect a lack of regulatory T cell expansion, a common limitation of wild-type IL-2 therapy. This aligns with the molecular design of the "no-alpha" mutein, which prevents binding to the IL-2 receptor alpha subunit—reducing regulatory T cell proliferation and supporting sustained immune activation [18,33]. Lastly there seemed to be no indication of increased vascular permeability in histopathological samples, as no major abnormalities were noted such as edemas. Notably, these histopathological findings were only observed following completion of three treatment cycles, suggesting a cumulative but controlled immune response consistent with

therapeutic goals.

4.7. Anti-drug antibodies

In relevance to anti-drug antibodies, the "no-alpha" mutein spurred anti-drug antibodies and only in treatment groups. At the end of the main study period, sex-combined data showed over 50 % of animals were positive for ADA and at the end of recovery 100 % were positive for ADA, with 0 % in control samples. It has been shown that many ADA responses from administration of aldesleukin and other IL-2 variants were deemed to be non-neutralizing [2,29,37]. Additionally, it was reported that development of IL-2 ADAs neither impacted the frequency nor duration of clinical responses in patients treated with recombinant IL-2 [29]. The induction of antibody formation in animals is not always predictive of a potential for antibody formation in humans [31]. As such, the presence of ADAs may not affect the effectiveness of the "no-alpha" mutein, however more in-depth clinical trials will be needed to fully understand the function of the derived ADAs.

4.8. CLS

Lastly, the primary drawback of HDIL-2 treatment is the occurrence of CLS which can cause hypotension and hemoconcentration, as well as lead to death without medical intervention [34,7]. In previous studies, the administrations of native IL-2 caused onsets of CLS symptoms of fever, chills, wheezing, amongst others in minutes to hours after the first dose [33]. Our study aimed to assess the presence of CLS, and we generally found no evidence after five days of dosing in animals at the 30x dose level. For example, CLS related signs such as abnormal breathing, hypotension and severe hemoconcentration were not observed in any of the animals. However, there was some evidence for CLS in the parameters of clinical pathology in animals that experienced the complete experimental duration. Both high dose males and females exhibited significantly higher neutrophil presence, that were both out of range. This hints at a mild inflammatory response, a marker of CLS, but it lacked corroborating evidence such as hemoconcentration or hypoalbuminemia.

This seems to suggest that high doses of the "no-alpha" mutein administered daily for a short time period may not cause CLS, making this mutein advantageous compared to the wildtype. It should be noted that future research should include more aspects like histopathology to focus on CLS. More attention should be focused on inflammatory markers of systemic CLS in mutein groups compared to wildtype. Including a component like this would allow a direct comparison to be made, increasing the accuracy of the results and conclusions drawn.

4.9. Summary

In summary, the "no-alpha" mutein was well tolerated in Sprague-Dawley rats when administered at dose levels of 1x (600 U/kg) and 10x (6000 U/kg) the proposed human dose across three 5-day dosing cycles. This conclusion is supported by comprehensive assessments, including clinical observations, body weight, food consumption, ophthalmologic exams, clinical pathology, gross pathology, and histopathology.

In contrast, administration at the 30x dose (18,000 U/kg) resulted in localized adverse clinical signs such as swelling, discoloration, and injection site necrosis. These effects were likely attributable to the irritant nature of the highly concentrated formulation. Midway through the study, these signs were mitigated by reducing the concentration of the formulation and increasing the injection volume to maintain the intended dose. Despite these localized reactions, the 30x dose was still considered tolerable, and the adverse effects were manageable and reversible. Importantly, no premature deaths or signs of capillary leak syndrome (CLS) were observed during either the in-life or recovery phases.

These findings suggest that the "no-alpha" mutein presents no observable adverse effects at the proposed human dose or even up to 10x that dose, with reversible and manageable effects at 30x. This is a promising result, particularly in the context of IL-2-based therapies.

Historically, IL-2 therapy has been limited by its toxicity profile. In the 90 s, only 15–20 % of patients responded to IL-2 treatment [4]. Those who did often experienced long-lasting, and in some cases even complete remission [26]. One of the main limitations of wild-type IL-2 is its tendency to promote the expansion of regulatory T cells, which suppress immune responses and reduce therapeutic efficacy [4,5]. This drawback required the use of high doses, further increasing the risk of severe side effects.

To address this, various IL-2 muteins have been developed, with "no-alpha" emerging as a leading candidate. This mutein was specifically engineered to reduce regulatory T cell expansion while promoting the proliferation of $CD8^+$ T cells and natural killer (NK) cells [4]. Previous work by Carmenate et al. [4] demonstrated that "no-alpha" significantly reduced lung nodules and melanoma cell lines more effectively than wild-type IL-2. Our findings in addition to those of Carmenate et al. [4], demonstrate that "no-alpha" is not only effective in targeting key immune effector populations and tumors, but also exhibits an improved safety profile. Rats successfully tolerated up to 10x the proposed human dose, without toxicity across multiple clinical and pathological endpoints.

To our knowledge, this is one of the few studies to incorporate multiple timepoints and endpoints to rigorously evaluate the toxicity of this IL-2 mutein. Uniquely providing a wholistic representation of the toxicity of the mutein. Our results support the notion that "no-alpha" may overcome one of the key barriers to IL-2 therapy—dose-limiting toxicity while eliminating CLS. Future directions for IL-2-based immunotherapy, particularly with muteins like "no-α," are promising. Especially in cancers such as melanoma and renal cell carcinoma, that are known to be historically responsive to IL-2. By limiting regulatory T cell expansion as well as enhancing CD8 + T cell and NK cell activation, "no-alpha" offers a safer and more targeted alternative to wild-type IL-2. Its improved tolerability at high doses enables more effective treatment regimens with fewer side effects. Additionally, the presence of anti-drug antibodies does not seem to affect the efficacy of the mutein, adding another benefit to the mutein. However additional research should be conducted to ensure the reliability of this notion, as it was a small component within our study. Continued evaluation in tumor-bearing models and clinical trials should be encouraged as they will be critical to establishing its full therapeutic potential. Given its dual benefit of enhanced efficacy and reduced toxicity, "no-alpha" represents a strong candidate for advancing IL-2-based cancer immunotherapy.

CRediT authorship contribution statement

Lozada Sum Lai: Methodology. **Bagshaw Richard:** Supervision, Project administration, Methodology. **Thambiahpillay Aaron:** Writing – original draft, Investigation, Data curation. **Ledon Nuris:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **NAGABASKARAN Gokulan:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis. **Licollari Albert:** Supervision, Project administration, Funding acquisition, Conceptualization. **Leon Kalet:** Methodology. **Rivas Gabriela:** Methodology.

Author statement

We sincerely appreciate the thoughtful and constructive feedback provided by the reviewers. We agree that the manuscript was in need of major revisions, and we have taken great care to address each comment thoroughly. To facilitate the review process, we have prepared a detailed response document in which we respond to each point raised. In this document, we explain our original reasoning where relevant,

outline the changes made to the manuscript in response to the feedback, and, in cases where changes were not made, provide a clear rationale for our decisions.

We are, of course, happy to make further revisions should any of our explanations not meet the reviewers' expectations. Thank you again for the opportunity to revise our work—we look forward to your feedback.

Ethical approval number

2021–59.AUP

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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