

A Preclinical Safety Study of Thyroid Hormone Instilled into the Lungs of Healthy Rats—an Investigational Therapy for ARDS[§]

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ABSTRACT

Acute respiratory distress syndrome (ARDS) is a severe, life-threatening form of respiratory failure characterized by pulmonary edema, inflammation, and hypoxemia due to reduced alveolar fluid clearance (AFC). Alveolar fluid clearance is required for recovery and effective gas exchange, and higher rates of AFC are associated with reduced mortality. Thyroid hormones play multiple roles in lung function, and L-3,5,3'-triiodothyronine (T3) has multiple effects on lung alveolar type II cells. T3 enhances AFC in normal adult rat lungs when administered intramuscularly and in normal or hypoxia-injured lungs when given intratracheally. The safety of a commercially available formulation of liothyronine sodium (synthetic T3) administered intratracheally was assessed in an Investigational New Drug Application-enabling toxicology study in healthy rats. Instillation of the commercial formulation of T3 without modification rapidly caused tracheal injury and often mortality. Intratracheal instillation of T3 that was reformulated and brought to a neutral pH at the maximum feasible dose of 2.73 μg T3 in 300 μl for 5 consecutive days had no clinically relevant T3-related adverse clinical, histopathologic, or clinical pathology findings. There were no unscheduled deaths that could be

attributed to the reformulated T3 or control articles, no differences in the lung weights, and no macroscopic or microscopic findings considered to be related to treatment with T3. This preclinical safety study has paved the way for a phase I/II study to determine the safety and tolerability of a T3 formulation delivered into the lungs of patients with ARDS, including coronavirus disease 2019-associated ARDS, and to measure the effect on extravascular lung water in these patients.

SIGNIFICANCE STATEMENT

There is growing interest in treating lung disease with thyroid hormone [triiodothyronine (T3)] in pulmonary edema and acute respiratory distress syndrome (ARDS). However, there is not any published experience on the impact of direct administration of T3 into the lung. An essential step is to determine the safety of multiple doses of T3 administered in a relevant animal species. This study enabled Food and Drug Administration approval of a phase I/II clinical trial of T3 instillation in patients with ARDS, including coronavirus disease 2019-associated ARDS (T3-ARDS ClinicalTrials.gov Identifier NCT04115514).

Introduction

Acute respiratory distress syndrome (ARDS) is a severe, life-threatening form of respiratory failure characterized by pulmonary edema, inflammation, and hypoxemia with a mortality rate of 40%. No specific molecular therapies are approved for ARDS, and the standard of care is still limited to mechanical ventilation, supplemental oxygen, and supportive care. The edema in ARDS results from epithelial and

endothelial damage with increased epithelial permeability and reduced alveolar fluid clearance (AFC). Fluid clearance from the alveoli is required for recovery and effective gas exchange, and higher rates of AFC are associated with reduced mortality (Matthay and Wiener-Kronish, 1990; Ware and Matthay, 2001; (Huppert and Matthay, 2017)Huppert and Matthay MA, 2017). The mechanism for AFC is active sodium absorption by the alveolar epithelium utilizing coordinated action of apical epithelial amiloride-sensitive sodium channels and basolateral sodium pump, Na⁺-K⁺ ATPase. Transalveolar fluid movement in lung alveoli is mediated primarily by the movement of sodium ions accompanied secondarily by chloride (Matthay et al., 2002). This sodium transport drives osmotic water absorption from the alveoli into the interstitium and pulmonary vasculature.

Thyroid hormones play multiple roles in lung development, function, and repair. It has recently been shown that

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ABBREVIATIONS: AFC, alveolar fluid clearance; ARDS, acute respiratory distress syndrome; AT2, alveolar type II; AUC, area under the curve; FDA, Food and Drug Administration; GLP, good laboratory practice; ESS, Experimental Surgical Services; IND, Investigational New Drug Application; MFD, maximum feasible dose; NBF, neutral buffered formalin; NCA, noncompartmental approach; RBC, red blood cell; T3, triiodothyronine; TK, toxicokinetic; Tmax, time postdose of maximum blood concentration of drug reached.

iodothyronine deiodinase 2, an enzyme that activates thyroid hormone, is correlated with disease severity in patients with idiopathic pulmonary fibrosis and that thyroid hormones can reduce fibrin deposition in both bleomycin and inducible transforming growth factor- β 1 models of pulmonary fibrosis in mice (Yu et al., 2018). It is of specific relevance that the thyroid hormone L-3,5,3'-triiodothyronine (T3) has multiple effects on lung alveolar type II (AT2) cells. T3 enhances AFC in adult rat lungs when administered intramuscularly, increasing AT2 cell Na,K-ATPase activity (Folkesson et al., 2000). When T3 was administered via intratracheal instillation, it rapidly increased AFC in both normal and hypoxia-injured rat lungs (Bhargava et al., 2008), suggesting that T3 can have direct effects on pulmonary tissues. Multiple studies have demonstrated that this augmentation of AFC by T3 occurs with upregulation of Na,K-ATPase sodium pump activity in AT2 cells. In vitro, this effect requires several secondary mediators, including mitogen-activated protein kinases, Src kinase, and PI 3-kinase that act in a post-translational fashion, increasing cell membrane Na,K-ATPase quantity (Jiang et al., 2001; Bhargava et al., 2007; Lei and Ingbar, 2011). At both physiologic and pharmacologic concentrations, T3 rapidly augments the alveolar epithelial cell's Na,K-ATPase sodium pump activity, increasing the AT2 cells' capacity to translocate fluid. These studies suggest that T3 directly instilled into human lungs can increase AFC and thereby improve oxygenation and decrease the need for prolonged mechanical ventilation. This route of potential therapy also may avoid potential side effects that might occur after systemic administration.

Liothyronine is a synthetic form of the naturally occurring thyroid hormone T3. The clinical formulation, liothyronine sodium injection (Triostat), is indicated for the intravenous treatment of myxedema coma, usually in conjunction with intravenous corticosteroid. T3 also has been administered intravenously to patients who are critically ill with a range of conditions in clinical trials without significant adverse effects, including severe sepsis, cardiomyopathy, and pericardiopulmonary bypass (Broderick and Wechsler, 1997; Marwali et al., 2017). However, no human or animal studies have assessed the safety of direct lung T3 treatments via airway instillation or nebulization. In consideration of a potential human phase I/II clinical trial of T3 treatment of ARDS via tracheal instillation, this study examined the pharmacodynamics and pharmacokinetic profile and safety of intratracheal T3 instillation of a Food and Drug Administration (FDA)-approved formulation of T3 in normal rats.

Materials and Methods

Good Laboratory Practice Compliance

The protocols and study designs were reviewed and approved as applicable by the Institutional Animal Care and Use Committee at the University of Minnesota for compliance with regulations prior to study initiation. This study was conducted according to the relevant standard operating procedures of the Center for Translational Medicine and Experimental Surgical Services. This nonclinical study was conducted in compliance with the Code of Federal Regulations Title 21 (Food and Drugs), Part 58, Good Laboratory Practice for Nonclinical Laboratory Studies, with the exception of the following: Microsoft Excel was used by the test facility to generate mean and S.D.

of numerical data. Microsoft Excel is not compliant with Code of Federal Regulations Title 21 Part 11 and Code of Federal Regulations Title 21 Part 58, but data integrity was maintained by burning a copy of the Excel worksheets upon which the descriptive statistics were performed to a read-only disk that was archived. Center for Translational Medicine-independent study director and quality assurance roles were provided by Experimental Surgical Services personnel. Histopathology was carried out by an outside, good laboratory practice (GLP)-compliant contract organization (Alizée, Pathology, Thurmont, MD). Clinical pathology analyses (hematology and clinical chemistry) were carried out by the Veterinary Medical Center, University of Minnesota, and the results were evaluated by a board-certified veterinary clinical pathologist.

Animals

This study was conducted in healthy male and female Sprague-Dawley rats (Hsd:Sprague DawleySD; Envigo). The rat model was used based on prior pharmacologic data from studies of thyroid hormone and associated receptors and physiologic responses in rat lung and on the similarities of hyperoxic lung injury in the rat with ARDS pulmonary edema in humans (Bhargava et al., 2008). The University of Minnesota is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International and registered with the United States Department of Agriculture to conduct research in laboratory animals. Animal studies conformed to National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals. National Institutes of Health publication 86-23. Revised 1985).

T3 and Control Solutions

T3 used for this study was liothyronine sodium injection (National Drug Code number 39822-0151-1; X-GEN Pharmaceuticals, Inc.) supplied as a sterile solution in 1-ml vials at a T3 concentration of 10 μ g/ml. Each milliliter of the T3 vehicle consisted of 6.8% by volume ethyl alcohol, 0.175 mg anhydrous citric acid, 2.19 mg of ammonium hydroxide, and USP-grade water. In preliminary studies we determined that rats do not tolerate intratracheal instillation of liothyronine sodium at a pH of >10.0 as it is supplied commercially. Therefore, the T3 and the T3 vehicle were adjusted to neutral pH (6.0–8.0) with sterile 1.0-N HCL (H9892; Sigma-Aldrich) added aseptically in a biosafety cabinet prior to intratracheal instillation into animals. pH was measured using pH test strips (8882, pH 4.5–10.0; Ricca). Because of neutrality of the solution falling on an exponential portion of the pH titration curve and the need to check pH frequently with the pH paper while maintaining sterility, this was a difficult procedure to perfect and required significant time and practice. In pilot studies, we determined that instillation of unmodified commercial formulations of liothyronine sodium rapidly caused tracheal injury and often mortality. The T3 solution volume increased approximately 10% after adjusting the pH to neutral with 1.0 N HCL, resulting in a final concentration of liothyronine sodium of approximately 2.73 μ g T3/300 μ l (9.17 μ g/ml). This solution was stored at 4°C and used for up to 26 hours after the pH adjustment procedure. The vehicle control solution was prepared in sterile, nonpyrogenic USP-grade water (9190, USP/EP Purified; Ricca) and contained per ml of solution 6.8% by volume alcohol (2716, USP Ethyl Alcohol; Decon), 0.175 mg anhydrous citric acid (27109, USP; Sigma-Aldrich), and 2.19 mg ammonia (JT Baker, ammonia solution, strong 27.0%–30.0%, N.F.–F.C.C.). The vehicle was also adjusted to neutral pH (6.0–8.0) with 1.0 N HCL as described above. Using aseptic technique, the vehicle was filter-sterilized and aliquoted into 21 sterile disposable tubes (5 ml each) and stored at 4°C, and a new tube was opened for each day of use. Normal saline (0.9% sodium chloride injection, USP; B. Braun Medical, Inc.) was stored at room temperature. A new package was opened for each day of use.

TABLE 1
Study design

Group	Toxicity Phase		TK Phase		Dose Volume
	Number of Toxicity Animals		Number of TK Animals		
	M	F	M	F	
1: T3 vehicle (control)	10 + 2 ^a	10 + 2 ^a	0	0	300 µl
2: Normal saline (control)	10 + 2 ^a	10 + 4 ^a	0	0	
3: T3 (test article)	10 + 2 ^a	10 + 2 ^a	12 + 2 ^b	12 + 2 ^b	
Totals	30 + 6	30 + 8	12 + 2	12 + 2	
	60 + 14		24 + 4		
Dosing frequency and duration (initial day of dosing = day 1)	*Dosed once daily for 5 days		Single dose		
Scheduled termination	1 day post-final dose		Day 1		
TK blood collection	NA		At designated time points		
Histology	Yes		NA		

F, females; M, males; NA, not applicable.

^aUse of spares: Two spare animals of each sex/toxicity group were dosed with the animals from each group so that they were available for replacement within the similar timeframe. An additional two females were dosed in group 2 because of early deaths experienced due to non-test material-related issues. The spare animals underwent terminal clinical pathology and gross necropsy evaluations.

^bUse of spares: Four additional unused spares were released from study at the direction of the study director.

Study Design

Details of the study design are shown in Table 1. Sixty animals, 10 animals/sex per group (FDA Redbook 2000 and direct correspondence with FDA regarding this program) plus two spare animals/sex per group, were anesthetized and dosed via intratracheal instillation with T3 or control solutions (normal saline or T3 vehicle) for 5 consecutive days. The saline group was included as a control and because in the clinical trial T3 will be diluted in saline prior to instillation into patients.

On the day after the last dose, a terminal blood collection was performed for clinical pathology, after which animals were euthanized, and gross examination of all organs was performed by a board-certified veterinary pathologist. Select tissues were collected for histopathology. Twenty-four animals in the toxicokinetic (TK) studies were anesthetized and dosed with a single intratracheal instillation of T3. Terminal blood collection was performed at two designated time points per animal up to 24 hours after administration for toxicokinetic evaluation. TK animals were euthanized without further evaluation after the final blood collection.

All animals received the same volume (0.3 ml) of either T3 or control solutions. In preliminary studies we determined that the maximum feasible dose (MFD) that could be delivered was dictated by the maximum volume (0.3 ml) that could be safely and reproducibly instilled over 5 days into the lungs of rats weighing 250–350 g. The actual doses delivered are reported as both microgram T3 per kilogram body weight and microgram T3 per gram wet lung weight in the *Results* section. In the toxicity studies, animals were 72–135 days of age at the time of initial dosing. Males weighed between 256.52 and 307.50 g with a mean \pm S.D. of 286.74 \pm 11.77 g, and females weighed between 250.46 and 299.01 g with a mean \pm S.D. of 260.64 \pm 10.34 g. The TK study animals were 68–144 days of age at the time of initial dosing. Males weighed between 261.23 and 316.19 g, and females weighed between 250.06 and 280.32 g.

In-Life Animal Care

Upon arrival rats were visually examined by trained staff and weighed, counted, sexed, and appropriately separated into housing boxes. Each animal received a metal ear tag containing an individual identifier prior to initial dosing. Animals were housed in Association for the Assessment and Accreditation of Laboratory Animal Care-accredited pens under sanitary conditions and were socially housed to provide enrichment and companionship. The temperature and

humidity of the housing area was monitored a minimum of once daily. Animals were acclimated with ad libitum access to irradiated food (2918; Teklad) and water for a minimum of 7 days prior to dosing initiation. Preconditioning was allowed during this period to acclimate the animals to the handling they would experience during weighing, examinations, and dosing procedures. Animals were not fasted for any of the procedures. Veterinary care was available throughout the course of the study. Observations on general health, including animal activity, appearance, food and water intake, mortality/moribundity, and other endpoints, were performed and recorded at least once daily from the time of enrollment on study until euthanasia by a trained technician, whereas detailed clinical observations were performed prior to initial dose, 1 day after initial dose, and at day of termination (see Table 1 in the Supplemental Data for a list of all clinical observation parameters). A veterinarian was notified of abnormalities in activity or appearance. To prevent bias regarding observations, health concerns, or treatments, veterinary and general animal care personnel were not informed of dose group distribution.

Dosing Procedure

Test and control materials were drawn into dosing syringes using aseptic technique. Using an 18G needle, 0.5 ml of air was drawn into a 1CC syringe followed by 0.3 ml (300 µl) of the solutions. Animals were anesthetized with a combination of ketamine, 40–200 mg/kg, and xylazine, 1–7 mg/kg intraperitoneal (i.p.), to effect. The dose was adjusted daily, as needed based on individual animal response and recovery. Depth of anesthesia was evaluated by toe pinch, and eye lubricant was applied to the eyes. An upright, inclined stand was used to support the animals in the desired position during the dosing procedure by suspending the animals from a soft, nonlatex rubber band at the top of the stand by their front incisors (Fig. 1A). Up to 20 µl of 2% lidocaine was applied topically to the back of the throat using a blunt gavage needle prior to intubation to minimize laryngeal spasms and facilitate tracheal placement. The animals were removed from the stand and positioned in prone position while the lidocaine took effect. All doses of test and control material were administered at the volume of 0.3 ml.

After allowing adequate time for lidocaine to take effect, the animals were again suspended on the apparatus, and a tracheal catheter (Clay Adams INTRAMEDIC Brand tubing cut at an angle, 427436, i.d. 1.19 mm, o.d. 1.70 mm) was inserted into the trachea by first enhancing the visibility of the larynx in the oral cavity with the

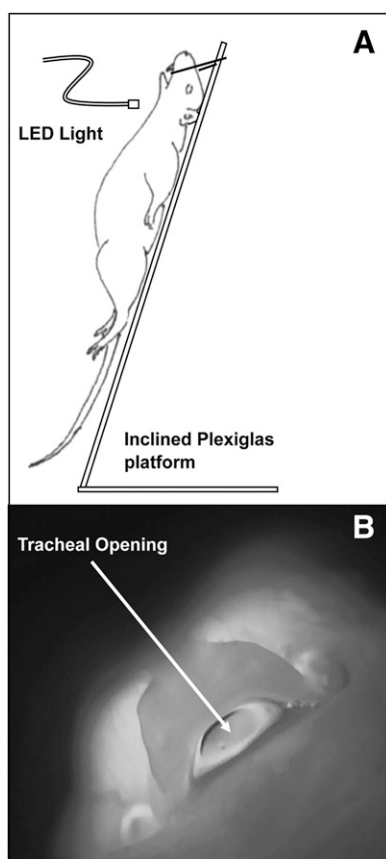


Fig. 1. (A) Setup for tracheal intubation and (B) visualization of tracheal opening through oral cavity. LED (light-emitting diode)

aid of an external light source directed at the throat (Fig. 1B). Holding the tongue aside with blunt forceps and gauze moistened with water further helped to make the airway visible. The catheter was advanced into the trachea to a predetermined depth of approximately 1.0 cm short of the branch point of the major bronchi (measured on a cadaver animal with the trachea and bronchi exposed). Catheter placement in the airway was verified by the fogging of a dental mirror placed at the opening of the catheter. The needle on the dosing syringe was then inserted into the catheter, and the T3 or control solutions followed by a bolus of air in the syringe was rapidly delivered in a 1–2-second interval. The air bolus administered after the test material facilitated administration of the fluid into the entire lung and ensured that fluid was not retained in the trachea or major bronchi, as confirmed in preliminary experiments using an Evans blue dye solution. The tracheal catheter was removed from the airway, and the animal was gently removed from the support apparatus. The animal was placed in a prone position on a heating pad with the chest elevated for

a minimum of 2 minutes after instillation. After 2 minutes, the animal was placed flat on a heating pad until fully recovered.

Terminal Procedures

Blood Collection for Clinical Pathology (Hematology and Clinical Chemistry). Blood samples from the toxicity study animals for clinical pathology were collected 1 day after the final (fifth) intratracheal dose. Animals were anesthetized with isoflurane 2%–5% and oxygen 1–1.5 l/min by inhalation anesthesia via nose cone as needed. For hematology, ≥ 0.5 ml whole blood was collected via the orbital sinus through plain or coated microhematocrit capillary tubes into K_2 EDTA collection tubes (BD microtainer, 365974) containing an additional 30 μ l of 2% EDTA solution (2854; Sigma-Aldrich) and kept at 4°C until same-day analysis. For serum chemistry, ≥ 0.75 ml whole blood was collected via the orbital sinus through uncoated capillary tubes into red top serum microtubes (41.1501.105, serum 1.3 ml; Sarstedt). For serum collection, tubes were maintained at room temperature for 30–60 minutes after collection and then centrifuged at 10,000g for 5 minutes at 4°C. The resultant serum was separated and stored at $\leq -70^\circ\text{C}$ if analysis were to occur the following day or kept at 4°C for same-day analysis. All samples were sent to the University of Minnesota Veterinary Medical Center clinical pathology laboratory for analysis. Parameters evaluated for hematology and clinical chemistry are provided in Tables 2 and 3, respectively. After blood collection, animals were euthanized with Euthasol ≥ 86 mg/kg i.p. to effect prior to necropsy. Assessment of the clinical pathology values was performed by Jill Schappa Faustich, DVM, DACVP, University of Minnesota.

Toxicokinetics

Blood Collection. Toxicokinetic study animals were anesthetized with combination of ketamine 40–200 mg/kg and xylazine 1–7 mg/kg i.p. to effect for dosing procedures and dosed intratracheally with liothyronine sodium injection as previously described. Mean concentrations were derived from three animals/sex per time point at 0 (predose), 15, and 30 minutes and 1, 2, 4, 6, and 24 hours postdose. Each animal was only bled twice (two time points/animal): one survival bleed and a terminal at euthanasia. Depending on the duration of time between dosing and the first or second blood collection time points, animals either had blood collected while still anesthetized under the injectable anesthetics, or, if recovered, they were anesthetized with isoflurane 2%–5% and oxygen 1–1.5 l/min by inhalation anesthesia via nose cone as needed to maintain adequate anesthesia depth (assessed by toe pinch). Topical proparacaine anesthetic ophthalmic solution was applied to each eye prior to performing the first blood collection and allowed time to take effect. Collection of serum samples for TK analysis was as described for serum chemistry samples above, and samples were stored at $\leq -70^\circ\text{C}$ until assayed. Animals were euthanized with Euthasol ≥ 86 mg/kg i.p. to effect after the final blood collection.

TABLE 2
Hematology parameters

RBC Count	
Hemoglobin conc. (HGB)	Red cell distribution width (RDW)
Free plasma HGB	White blood cell (WBC) count
Hematocrit (HCT)	Neutrophil Segmented count (NEUT absolute and relative)
Mean corpuscular volume (MCV)	Neutrophil band count (BAND absolute and relative)
Mean corpuscular hemoglobin (MCH)	Lymphocyte count (LYMPH absolute and relative)
Mean corpuscular hemoglobin conc. (MCHC)	Monocyte count (MONO absolute and relative)
Reticulocyte Count (Retic absolute and relative)	Eosinophil count (EOS absolute and relative)
Platelet count (Plt and PCT absolute and relative)	Basophil count (BASO absolute and relative)
Mean platelet volume (MPV)	Mast cells (absolute and relative)
Platelet distribution width (PDW)	Unclassified cell count (absolute and relative)

TABLE 3
Serum chemistry parameters

Blood Urea Nitrogen (BUN)	
Creatinine (Creat)	Osmolality (Osmol)
Calcium (Ca)	Anion gap (An Gap)
Phosphorus (Phos)	Bilirubin, total (T.Bili)
Magnesium (Mg)	Alkaline phosphatase (ALP)
Protein (TP)	γ -Glutamyl transferase (GGT)
Albumin (Alb)	Alanine transferase (ALT)
Globulin (Glob)	Aspartate transferase (AST)
Alb/Glob ratio	Creatine kinase (CK)
Sodium (Na)	Glucose (Gluc)
Chloride (Cl)	Cholesterol (Chol)
Potassium (K)	Amylase
Bicarbonate (HCO_3)	Lipemia icterus hemolysis (LIH)

Bioanalytical Procedure. For assessment of serum T3 levels, samples were sent to the Fairview University of Minnesota Medical Center East Bank Diagnostic Laboratory for analysis, a clinical laboratory certified by Clinical Laboratory Improvements Amendments and College of American Pathologists. Prior to sending serum samples to the analytical laboratory, each sample was diluted 1:4 or 1:8 in normal (0.9%) saline. These dilutions, which were determined in preliminary studies, ensured that sample total T3 concentrations would fall within assay range (10–460 ng/dl). Samples were analyzed by a chemiluminescence assay for total T3.

Data Analysis. Observational data were summarized using descriptive statistics. Continuous data were summarized in terms of mean and S.D. Significance testing was not performed. The clinical relevance of any differences noted in clinical pathology endpoints was determined solely by the clinical pathologist who was blinded to the treatment groups.

TK Analysis. Toxicokinetic parameters were estimated using Phoenix 64 WinNonlin pharmacokinetic software version 7.0 (PharsightCorp., MountainView, CA). A noncompartmental approach (NCA) consistent with the route of administration was used for parameter estimation. All parameters (Table 4) were generated from mean T3 concentrations in serum from all time points unless otherwise stated. Parameters were estimated using sampling times relative to the start of each dose administration. The raw data were converted to nanogram per milliliter of serum by dividing the nanogram per deciliter values by 100 and then multiplying by the dilution factor for that sample, which was either 4 or 8. Values below the limit of quantification were calculated as zero.

Calculation of arithmetic mean and S.D. for the matrix concentration data was performed/replicated in Microsoft Excel for reporting purposes. In addition to parameter estimates from mean concentration versus time curves, the S.E. of the AUC(0–t) and

C_{\max} by dose group, day, and sex (as appropriate) was generated using WinNonlin.

C_{\max} and time postdose of maximum blood concentration of drug reached (Tmax) were obtained by inspection of the data. Since measurable endogenous compound is present based on the observed concentration at time zero, a baseline subtraction was performed. Using the mean concentration data, the concentration at time zero was subtracted from the remaining concentrations for male and female animals. The area under the curve (AUC) of the baseline subtracted concentrations was calculated using the linear trapezoidal rule. Since the 24-hour concentration in both male and female animals had approximately returned to the baseline (predose) concentration, these observations were ignored in calculations for the AUC and half-life. The terminal elimination half-life was calculated from the last three observations at times 2, 4, and 6 hours. WinNonlin NCA performs linear regression on the logs of the concentrations. The uniform weighting scheme was selected. The default regression algorithm for NCA does not use C_{\max} in the calculation of half-life, even if it appears to be part of the log-linear profile, nor does it provide any half-life based on only two observations. The default regression for the male animals was used. However, for the female animals, the concentration at time 2 hours was also the C_{\max} value. Since it appeared to fall on the regression line of all three concentrations (adjusted $R^2 = 1.0$), it was included in the calculation of the half-life. Parameters were evaluated as appropriate at the discretion of the evaluator. Results are provided as individual values and include graphing of mean and S.E. using Microsoft Excel and WinNonlin per appropriate groups when possible.

Necropsy Procedures

Gross Pathology. Toxicity study animals that were euthanized at scheduled termination or that were found dead or euthanized prior to scheduled termination were subjected to an extensive necropsy performed by a board-certified veterinary pathologist. The necropsy included an examination of the animal carcass and musculoskeletal system; external surfaces and all its orifices; and cervical, thoracic, abdominal, and pelvic regions, cavities and contents. Eyes were not examined because of terminal orbital blood collection methods.

Histopathology. The primary target tissues assessed in this study for histopathologic changes included the lungs, the trachea-bronchi branch point, and the tracheobronchial lymph nodes. The intact heart-lung pluck, including all target tissues noted above, was removed from the animal intact. The heart-lung pluck was weighed and photographed, and the lungs were then perfusion-inflated via the trachea with 10% neutral buffered formalin (NBF). For inflation, an 18-g butterfly catheter connected to a reservoir of 10% NBF was

TABLE 4
TK parameters estimated

Parameter	Description of Parameter
C_{\max}	The maximum observed arithmetic mean conc. of T3 measured after dosing.
C_{\max}/D	The C_{\max} divided by the dose administered.
Tmax	The time after dosing at which the maximum observed arithmetic mean conc. of T3 was observed.
AUC(0–t)	The area under the T3 arithmetic mean conc. vs. time curve from time zero the time after dosing at which the last quantifiable concentration of the drug was observed estimated by the linear or linear/log trapezoidal method.
AUC(0–t)/D	The AUC(0–t) divided by the dose administered.
When data permitted, the slope of the terminal elimination phase of each arithmetic mean concentration vs. time curve was determined by log-linear regression, and the following additional parameters were estimated:	
Additional parameters estimated	
Parameter	Description of Parameter
$t_{1/2}$	The apparent terminal elimination half-life.
AUC(0–inf)	The area under the arithmetic mean concentration vs. time curve from time zero to infinity.
AUC(0–inf)/D	AUC(0–inf) divided by the dose administered.
CL	Clearance: the apparent volume of plasma cleared of T3 per unit time after intravenous dosing.
Vd	The apparent volume of distribution of T3, determined from the terminal elimination phase after intravenous dosing.

TABLE 5

Calculated T3 dose

Toxicity phase 1 ml T3 (10 µg/ml) diluted with ~100 µl 1.0-N HCl to pH, 10 µg in 1.10 ml = 2.73 µg in 300-µl dose.

Calculated Dose	Group 3 (T3) All	Group 3 (T3) Males	Group 3 (T3) Females
µg T3/g wet lung weight [avg. (S.D.)]	1.57 (0.61)	1.50 (0.15)	1.63 (0.14)
µg T3/kg body weight [avg. (S.D.)]	10.00 (0.64)	9.45 (0.33)	10.55 (0.25)

inserted into the trachea, and the lungs inflated for 2 minutes at a constant pressure of ~20–25 cm, after which the trachea was tied off with suture to maintain inflation of the lungs during fixation. The entire heart-lung pluck was then immersion-fixed in 10% NBF. Prior to further processing for histology, the heart, trachea, and any other adherent tissues were removed from the lungs and weighed. This weight when subtracted from the weight of the heart-lung pluck taken at necropsy provided the wet lung weight used in subsequent calculations of actual dose delivered. Nontarget tissues, including the brain, heart, liver, spleen, pancreas, kidneys, and adrenal glands, were evaluated for gross lesions. The nontarget organs were collected whole except for the liver, in which a representative specimen was collected from the anterior right lobe and stored in 10% NBF for potential future analysis. Histologic processing and evaluations were performed by Dr. Joan Wicks, DVM, PhD, DACVP, Alizée Pathology, LLC, Thurmont, MD. Histopathologic changes in the pulmonary tissues of all animals were evaluated and scored for incidence of findings for the following conditions: alveolar macrophage foci, alveolar histiocytosis, chronic bronchioalveolar inflammation, autolysis, acute congestion, acute edema, and acute hemorrhage. The pulmonary tissues examined included the left lobe, right cranial and caudal lobe, middle and accessory lobes, tracheobronchial lymph nodes, and the trachea, including the bifurcation of the major bronchioles.

Results

Dose Calculations of T3 Delivered. The doses of T3 administered to the lung in the toxicology study were calculated based on body weight and on wet lung weight on the initial day of administration (day 1) and are detailed in Table 5. FDA guidance stated that microgram T3 per gram wet lung weight is the preferred measure of actual dose delivered for this route of administration.

In-Life Observations. Porphyrin staining is a nonspecific response to stress in rats, including but not limited to anesthetic events, stressful handling, transport stress, or other procedures. Both male and female rats prior to and throughout dosing were observed to have slight/mild porphyrin staining (oral exudate of bile-derived porphyrin). None of the observations regarding porphyrin staining can be directly attributed to treatment with T3.

During daily observations, there were three postdose reports (out of 345 doses in the toxicity study) of hypersalivation, including one with labored breathing. There were no other notable clinical observations or complications throughout the course of the study with the exception of seven unscheduled deaths (described below). All surviving animals were in apparent good health prior to scheduled termination.

On average, body weights for all groups (including the vehicle groups) declined within the first 2 days of dosing by 5.5 ± 1.0 g for males (about 1.9%), and 4.4 ± 1.3 g for females (about 1.7%). By the time of termination, body weights rebounded to greater than the predose weights with the exception of T3 females that still weighed slightly less than their predose weight. Prior to termination, male toxicity study

animals weighed between 267.17 and 309.60 g with a mean \pm S.D. of 285.28 ± 11.10 g. Female toxicity study animals weighed between 240.11 and 278.44 g with a mean \pm S.D. of 258.24 ± 9.79 g.

Five out of a total of 74 animals dosed in the toxicity study, four in the saline control group, and one in the vehicle control group died during recovery from anesthesia. These deaths occurred approximately 30–60 minutes after administration of the first dose (day 1) and were judged by the veterinary pathologist conducting the postmortem exam to have been caused by exposure to excessive temperatures during recovery from anesthesia on heating pads. There were also two T3 test animals (out of 24 total in the toxicity study) that died after dose administration. For one of these deaths, gross pathology findings indicated it to be due to colon impaction, and in the other animal the cause was undetermined but thought to be due to injury during the instillation procedure. Of all animals dosed with the test or control articles, only two of seven early deaths were animals treated with T3. In spite of these losses, the planned minimum number of animals of each sex per group as outlined in the study design was achieved since additional animals were included in each dose group in the original study design as replacement animals.

Clinical Pathology. In the toxicity assessment experiments, comprehensive hematology and serum chemistry evaluations were performed on all animals at the scheduled termination with the exception of one animal that did not receive hematology analysis because of a clotted sample.

The effects of T3 treatment on hematologic and clinical chemistry values were assessed by the clinical pathologist who determined that there were no clinically relevant differences. Several minor, nonclinically relevant differences were observed and are summarized in Table 6. Female rats had unchanged hemoglobin but a mildly increased reticulocyte count and degree of polychromasia in the T3 group compared with the control groups (likely clinically irrelevant). Male rats had no differences in hematologic values with T3 treatment. The only difference in chemistry values with T3 treatment in the female rats was a slight difference in the mean albumin level (likely not clinically relevant). Male rats had only a slight difference in the mean globulin level, which was again considered clinically irrelevant. There were no other notable changes in the clinical pathology laboratory data (see Tables 2 and 3 in the Supplemental Data for a summary of assay results for all hematology and clinical chemistry parameters measured). In preliminary, non-GLP studies, neither single intravenous instillations of T3 nor 5 days of intratracheal delivery of 3.0 µg neutral-pH T3 had any acute effects on physiologic parameters [e.g., respiratory rate, O₂ saturation, or heart rate (unpublished data)].

Gross Pathology and Necropsy. The animal necropsy and gross pathology observations were performed by board-certified veterinary clinical pathologists. Aside from the early deaths discussed above, all toxicity study animals were

TABLE 6
Clinical pathology key findings

Parameter	Units	Mean \pm S.D.		
		Saline	T3 Vehicle	T3
Reticulocyte count (females)	$10^3/\mu\text{l}$	0.28 ± 0.04	0.31 ± 0.05	0.42 ± 0.04
Polychromasia (females)	%	3.7 ± 0.8	3.6 ± 0.8	5.5 ± 1.4
Albumin (females)	g/dl	3.49 ± 0.17	3.48 ± 0.16	3.21 ± 0.12
Globulin (males)	g/dl	2.81 ± 0.12	2.89 ± 0.13	2.63 ± 0.12

euthanized at the scheduled euthanasia time point. Necropsy was performed on all animals on the day of death with the exception of the animals that died during recovery from anesthesia, for which necropsy was performed on the day after death. During the necropsy procedures, there were no findings of differences in the weight of the lungs related to

treatment with T3, and no gross lesions were detected in the lungs of any of the animals in this study. Intra-abdominal lesions were detected in a total of four animals from among the three treatment groups that probably were associated with terminal intraperitoneal injections of anesthesia drugs. No evidence of toxicity related to drug administration was

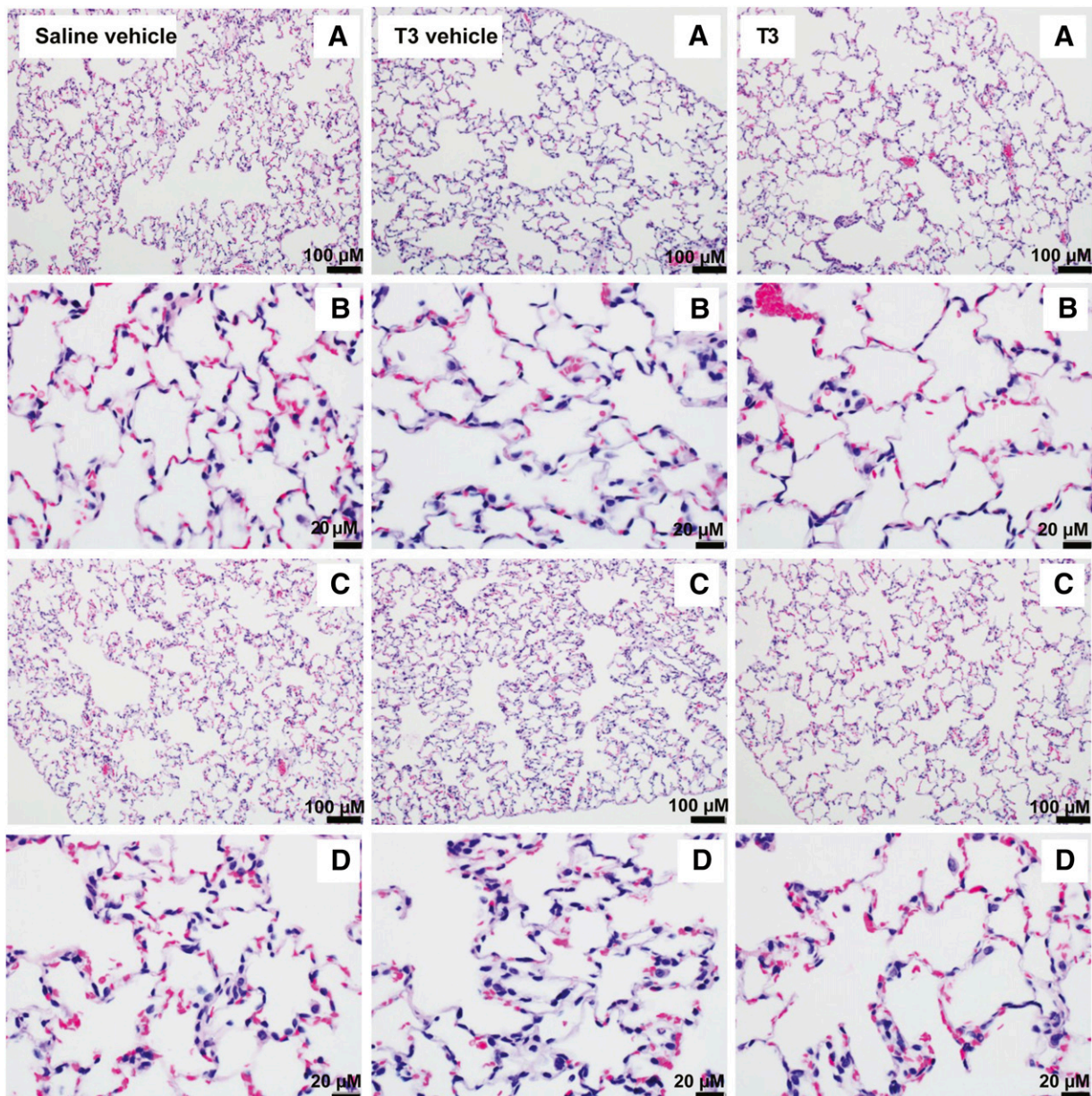


Fig. 2. Representative histology of lungs. (A) Apical region of left lung lobe (100 \times), (B) apical region of left lung lobe (400 \times), (C) basal region of left lung lobe (100 \times), (D) basal region of left lung lobe (400 \times).

TABLE 7

T3 detected in serum in single-dose TK study

	T3 (ng/ml) Mean and S.E.M.							
	0	15 min	30 min	1 h	2 h	4 h	6 h	24 h
Male	1.16	2.24	3.48	8.44	6.72	6.08	3.96	0.76
	1.28	2.52	3.76	6.84	6.92	4.52	3.24	0.80
	1.20	2.44	3.36	8.16	7.28	5.36	3.64	0.72
Mean	1.21	2.40	3.53	7.81	6.97	5.32	3.61	0.76
S.E.M.	0.04	0.08	0.12	0.49	0.16	0.45	0.21	0.02
Female	1.44	2.56	4.92	15.60	17.68	12.32	7.52	1.84
	0.88	3.20	5.16	16.08	18.32	11.48	6.92	1.24
	1.04	3.24	7.44	13.68	16.96	10.00	7.64	1.60
Mean	1.12	3.00	5.84	15.12	17.65	11.27	7.36	1.56
S.E.M.	0.17	0.22	0.80	0.73	0.39	0.68	0.22	0.17

observed grossly, and no clinically relevant abnormalities were noted during necropsy that were related to the administration of test or control articles.

Histopathology. Histologic evaluation demonstrated no microscopic differences in lung structure between the treatment groups apart from some macroscopic findings consistent with agonal changes, autolysis, and/or changes related to the intraperitoneal injections or intratracheal instillations, which were present in all groups. No abnormalities of the airway or alveolar tissues were seen in any of the rats treated with the pH-adjusted instillates. Initial trials with non-pH-adjusted T3 (pH ~10.7) resulted in death of the animals shortly after instillation, apparently due to respiratory distress (unpublished data). Representative photomicrographs of pulmonary tissues from the three treatment groups are shown in Fig. 2. The photomicrographs were taken of randomly selected histopathology sections of lung tissues by a pathologist who was blinded to the different treatment groups. See Table 4 in the Supplemental Data for a summary of histopathology scoring data for all pulmonary tissues.

With regard to the seven unscheduled deaths noted above (one in the vehicle control group, four in the saline control group, and two in the T3 group), all of the animals died shortly after intratracheal instillation or were found dead postdose. Macroscopic and microscopic findings in all animals were consistent with autolysis and/or agonal change. The cause of death in the T3-treated animals likely was related to the instillation procedure in one animal and colon impaction in the other and was not test article-related. There were no macroscopic or microscopic findings in any of the end organs examined (lungs, brain, heart, liver, spleen, pancreas, kidneys, and adrenal glands) in any of the remaining animals in the study that were determined to be related to treatment with T3.

Toxicokinetic Study. T3 was successfully quantified for all of the TK samples submitted. All reported values were within the limits of quantification for the assay (0.1–4.6 ng/ml).

The time course of serum T3 levels and calculated TK parameters after single dose T3 administration is presented in Tables 7 and 8 and in Fig. 3 (mean \pm S.D.). Noncompartmental analysis of TK samples was performed on diluted serum samples from all 24 animals that received a single T3 dose (Table 8). Female rats had greater serum T3 concentrations than males, likely because of the somewhat lower body weights of the females. The C_{max} was 16.5 ng/ml in females and 6.60 ng/ml in males, and the AUC was 89.70 ng*h/ml in females and 36.34 ng*h/ml in males.

In a non-GLP pilot study, intratracheal administration of 3.0 μ g T3 resulted in a C_{max} that was \sim 1/17th and an AUC that was \sim 1/5th of what we saw after intravenous administration of 3.0 μ g T3 (Fig. 4).

Discussion

The primary objective of this IND-enabling preclinical study was to assess the safety, tolerability, and toxicokinetics of T3 instilled directly into the lungs of rats. Using healthy rats, T3, control vehicle, or saline was administered intratracheally at the maximum feasible dose on 5 consecutive days. Although in pilot studies we encountered the surprising finding that instillation of commercially available liothyronine sodium led to rapid mortality of the animals, with instillation of the pH-adjusted, reformulated T3, no clinically relevant adverse T3-related clinical, histopathologic, or laboratory abnormalities were observed. There were no unscheduled deaths that could be attributed to the test or control treatments, no differences in the lung weights, and no macroscopic or microscopic findings considered to be related to treatment with T3. In conclusion, there were no adverse events or clinically significant complications related to instillation of the test or control materials. We expected that daily handling, intravenous anesthesia, and intubation procedures would affect appetite and weight gain and that the extent of this anesthesia-mediated effect would be highly variable among individual animals depending on their depth of anesthesia, rate of recovery, and resumption of normal feeding and drinking behaviors. None of the weight loss observed reached a level (10%–15%) considered concerning according to the Institutional Animal Care and Use Committee guidelines.

In general, the clinical pathology changes noted between the T3 group and control groups were considered to be not clinically relevant and not directly related to the administration of T3. The difference in the mean serum globulin level

TABLE 8

Noncompartmental analysis of TK samples

Sex	C_{max} (ng/ml)	C_{max_D} (ng/ml per microgram)	T_{max} (h)	HL_Lambda_z (h)	AUClast (h*ng/ml)
F	16.53	6.12	2.00	2.85	64.08
M	6.60	2.44	1.00	3.17	25.38
Sex	AUCINF_obs (h*ng/ml)	AUCINF_D_obs (h*ng/ml per microgram)	Cl_F_obs (ml/h)	Vz_F_obs (ml)	
F	89.70	33.22	30.10	123.60	
M	36.34	13.46	74.29	339.44	

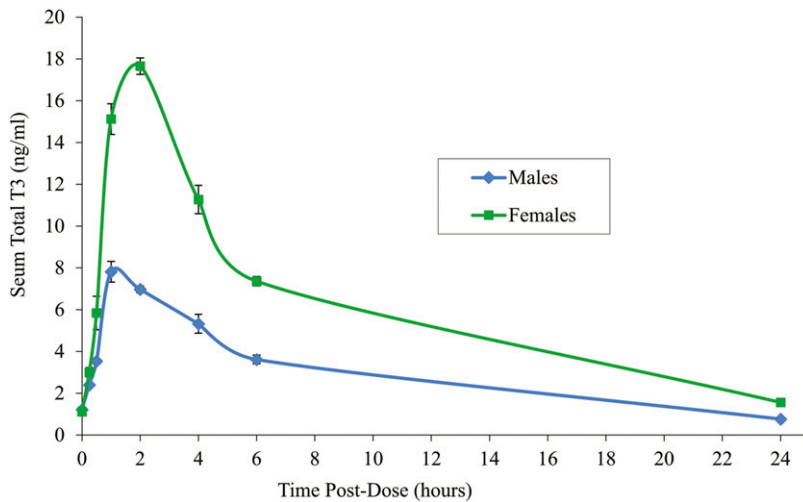


Fig. 3. Serum T3 concentration (mean \pm S.D.) vs. time after intratracheal instillation of 2.7 μ g T3 in 300 μ l, pH 7.5. Samples were analyzed by a chemiluminescence assay for total T3. $N = 3$ animals/sex per time point.

between the male T3 and control groups was minimally different and considered clinically irrelevant and likely due to either a change in food intake or a change in intravascular volume status. The clinical relevance of the slightly higher reticulocyte count in the T3 female rat group is somewhat difficult to interpret considering the absence of a concurrent decrease in hematocrit, hemoglobin, red blood cell (RBC) count, and abnormal RBC morphology. This result could be indicative of an increase in RBC turnover but, in the absence of anemia, is likely to be clinically irrelevant. The lower serum albumin level in the female T3 group was minimally different. There were no other notable biochemical abnormalities in these rats, thus this finding is likely to be clinically irrelevant.

In all study animals, the test materials were successfully administered with minimal test material-related adverse events or complications. Out of a total of 98 animals that received at least one dose, seven animals in the toxicity study (one vehicle control, four saline controls, and two T3 animals) and zero animals in the toxicokinetic study died before the end of the study. None of these deaths were likely related to treatment with the test article.

Peak serum levels of T3 occurred at one and two hours after instillation for males and females, respectively. The female rats had consistently higher serum T3 levels than the males at corresponding time points. All animals regardless of body weight received the same dose of T3. On average, females had lower body weights than males and therefore received a slightly higher calculated dose per body weight and a slightly higher dose per calculated wet lung weight than males. In addition, in preliminary studies female rats had a larger average lung weight/body weight ratio than males, suggesting that female rats may have a relatively larger lung surface area for a given body weight, potentially allowing greater absorption of T3 into the systemic circulation (unpublished data).

The MFD of T3 that could be administered in this study, ~ 2.73 μ g, was limited by both the volume of instillate that could be safely and reproducibly administered intratracheally to rats (0.3 ml) and by the concentration of the commercially available T3. The inability to administer higher T3 doses prevented us from establishing a maximum tolerated dose and from collecting some of the data that would typically be generated in a definitive preclinical safety study (e.g., dose response, target organs, recovery). However, the MFD is

essentially a no-observed-adverse-event-level dose and on a microgram T3 per gram wet lung weight basis is approximately 300-fold higher than the starting dose for the planned first-in-human clinical trial.

T3 was administered intratracheally in this study because it was specifically designed to support a clinical trial in which T3 would be instilled directly into the lungs of intubated patients with ARDS. The FDA requires that whenever possible drugs used in IND-enabling studies are to be administered by the identical route that will be used in the clinic. Although aerosol administration is being explored for patients either with or without intubation, a preclinical study in which T3 was administered by a route other than direct instillation would not have provided the safety data needed to support the planned first-in-human clinical trial. In addition, nebulized drugs are less likely to be delivered to lung regions that have diminished ventilation due to injury and/or alveolar flooding that is frequently observed in the lungs of patients with ARDS.

As an IND-enabling safety study, this preclinical study was conducted to be GLP-compliant, which can be challenging in an academic environment. The University of Minnesota is fortunate to have two organizations that routinely conduct GLP-compliant studies—the Center for Translational Medicine and Experimental Surgical Services (ESS). The Center

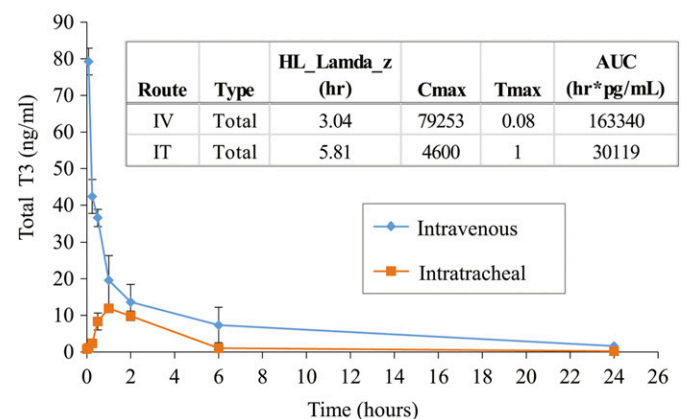


Fig. 4. T3 exposure after intravenous vs. intratracheal administration of 2.7 μ g T3 in 300 μ l, pH 7.5. Samples were analyzed by a chemiluminescence assay for total T3. $N = 3$ animals/sex per time point.

for Translational Medicine was created in 2007 and conducts feasibility, efficacy, pharmacokinetic, and IND-enabling safety studies to advance promising preclinical therapeutic candidates into clinical and commercial development. ESS is a leading preclinical research center within the Department of Surgery that has conducted GLP safety studies for medical devices for more than 30 years. GLP studies conducted by the Center for Translational Medicine are done in partnership with ESS, which has an in-house quality assurance unit. Working with ESS, the Center for Translational Medicine has directed or designed IND-enabling studies for eight therapeutic candidates, of which four have advanced to a proof-of-concept clinical trial or been licensed for continued development.

In summary, intratracheal instillation of T3 in a reformulated, pH-adjusted solution at the maximum feasible concentration for up to 5 consecutive days had no clinically relevant impact on vital signs, hematologic and chemical values, or lung and other organ histology. The study provided preclinical safety and tolerability data necessary to secure FDA approval for a phase I/II clinical trial of T3 instillation in patients with ARDS, including coronavirus disease 2019–associated ARDS (T3-ARDS ClinicalTrials.gov Identifier NCT04115514).

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Authorship Contributions

Participated in research design: Flory, Norris, Rich, Ingbar, Schumacher.

Conducted experiments: Flory, Norris, Larson, Coicou, Koniar, Mysz.

Contributed new reagents or analytic tools: Flory, Coicou.

Performed data analysis: Flory, Norris, Rich, Ingbar, Schumacher.

Wrote or contributed to the writing of the manuscript: Flory, Norris, Larson, Coicou, Koniar, Mysz, Rich, Ingbar, Schumacher.

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Online Data Supplement:

Title: [A Preclinical safety study of thyroid hormone instilled into the lungs of healthy rats – an investigational therapy for ARDS](#)

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David H. Ingbar², Robert J. Schumacher¹

Supplementary Table 1. In life Observations/Assessments & Health Monitoring

Activity	Frequency
Handling & Restraint Pre-Conditioning	SID beginning within 2 days after arrival
Body Weight (grams)	Within 1 days prior to and/or on the day of initial dose. Minimally 2-3 times/week during dosing Day of term
Cage Side Observations <ul style="list-style-type: none"> • Mortality/Alive or Dead • Defecation • Urination • Behavior/vocalization • Posture/Attitude/Activity • Food available • Water available, bottle intact • Bedding sufficient 	Minimally SID beginning within 3 days prior to initial dose. Day of term
Detailed Clinical Observations¹ <ul style="list-style-type: none"> • Posture/Attitude • Integument/Hair • Eyes • Ears • Nose • Respiratory • Musculoskeletal • Urogenital • Gastrointestinal • Neurological 	At least once prior to initial dosing At least once after initial dose Day of Term

Supplemental Table 2. Summary of Hematology Raw Data

Analyte	Males (n=11/treatment), Mean (SD)				Females (n=11/treatment), Mean (SD)			
	Reference Range ¹	T3 Vehicle	Saline Vehicle	T3	Reference Range**	T3 Vehicle	Saline Vehicle	T3
WBC (x10 ³ /μL)	8.7-14.7	11.31 (2.28)	12.6 (2.91)	12.48 (1.91)	6.6-12.2	10.31 (1.27)	9.07 (1.83)	9.82 (1.62)
RBC (x10 ⁶ /μL)	6.7-7.7	8.29 (0.47)	8.41 (0.37)	8.3 (0.36)	5.3-8.3	7.95 (0.31)	8.04 (0.28)	7.87 (0.2)
HGB (g/dL)	13.6-15.8	15.24 (0.71)	15.33 (0.49)	15.22 (0.46)	11.0-16.8	14.71 (0.54)	14.81 (0.43)	14.4 (0.4)
HCT (%)	47.3-55.5	49.63 (2.36)	49.97 (1.89)	49.23 (1.48)	36.5-57.3	46.8 (1.69)	46.98 (1.7)	46.45 (0.94)
MCV (fL)	69.8-73.6	59.95 (1.4)	59.45 (1.57)	59.4 (1.66)	65.9-71.7	58.93 (1.19)	58.44 (1.39)	59.11 (2.07)
MCH (pg)	19.8-21.2	18.42 (0.36)	18.22 (0.47)	18.34 (0.5)	19.6-21.2	18.52 (0.3)	18.41 (0.33)	18.33 (0.7)
MCHC (g/dL)	27.9-29.3	30.75 (0.52)	30.65 (0.49)	30.89 (0.28)	28.8-30.4	31.44 (0.32)	31.54 (0.38)	31.01 (0.72)
Retic (#)	NR	0.25 (0.13)	0.2 (0.04)	0.24 (0.03)	NR	0.31 (0.05)	0.28 (0.04)	0.42 (0.04)
Retic (%)	NR	3.06 (1.87)	2.44 (0.59)	2.95 (0.42)	NR	3.88 (0.7)	3.5 (0.4)	5.31 (0.62)
RDW (%)	NR	10.83 (0.62)	10.75 (0.33)	11.02 (0.49)	NR	11.63 (0.43)	11.37 (0.33)	11.69 (0.36)
Plt (x10 ³ /μL)	NR	966.73 (239.37)	867.82 (75.28)	857.8 (196.22)	NR	985.18 (256.59)	844.73 (195.28)	904.18 (187.66)
MPV (fL)	NR	7.4 (0.51)	7.38 (0.31)	7.7 (0.22)	NR	7.58 (0.27)	7.88 (0.58)	7.69 (0.35)
PDW (%)	NR	55.36 (5.1)	52.89 (3.52)	56.95 (6.53)	NR	49.29 (2.04)	52.83 (3.11)	54.34 (6.76)
PCT (%)	NR	0.72 (0.21)	0.64 (0.07)	0.66 (0.15)	NR	0.75 (0.2)	0.66 (0.14)	0.7 (0.15)
MPC (g/dL) ²	NR	21.22 (1.28)	21.02 (0.75)	20.72 (0.86)	NR	20.37 (0.89)	19.72 (1.14)	20.25 (0.65)
MPM (pg) ²	NR	1.41 (0.03)	1.4 (0.03)	1.42 (0.05)	NR	1.4 (0.05)	1.39 (0.05)	1.4 (0.04)
% NEUT	6.8-11.4	13.08 (5.19)	10.91 (2.45)	10.3 (3.99)	3.1-7.9	13.92 (4.29)	14.72 (5.16)	14.8 (6.24)
% BAND	NR	0 (0)	0 (0)	0 (0)	NR	0 (0)	0 (0)	0 (0)
% LYMPH	75.7-86.5	82.9 (5.59)	85.18 (2.75)	86.01 (4.54)	78.1-91.5	81.47 (5.29)	79.73 (5.93)	78.63 (7.49)
% MONO	3.6-8.4	2.9 (2.07)	3 (1.41)	2.99 (1.48)	2.2-12.2	3.08 (1.44)	3.19 (1.41)	4 (1.77)
% EOS	1.1-6.5	0.64 (0.64)	0.64 (0.79)	0.5 (0.5)	0.0-5.1	1.18 (0.82)	2.09 (1.84)	2.36 (0.98)
% BASO	0.0-0.2	0.46 (0.51)	0.27 (0.45)	0.2 (0.4)	0.0-0.2	0.38 (0.5)	0.27 (0.44)	0.18 (0.38)
# NEUT (x10 ³ cells/μL)	NR	1.46 (0.69)	1.35 (0.37)	1.28 (0.51)	NR	1.45 (0.51)	1.31 (0.55)	1.44 (0.59)
# BAND (x10 ³ cells/μL)	NR	0 (0)	0 (0)	0 (0)	NR	0 (0)	0 (0)	0 (0)
# LYMPH (x10 ³ cells/μL)	NR	9.43 (2.21)	10.76 (2.57)	10.75 (1.76)	NR	8.39 (1.1)	7.26 (1.64)	7.73 (1.5)
# MONO (x10 ³ cells/μL)	NR	0.3 (0.17)	0.4 (0.24)	0.35 (0.14)	NR	0.31 (0.14)	0.31 (0.19)	0.39 (0.19)
# EOS (x10 ³ cells/μL)	NR	0.07 (0.07)	0.07 (0.08)	0.07 (0.07)	NR	0.13 (0.09)	0.17 (0.13)	0.24 (0.13)
# BASO (x10 ³ cells/μL)	NR	0.05 (0.05)	0.03 (0.05)	0.03 (0.06)	NR	0.04 (0.05)	0.03 (0.04)	0.02 (0.04)
RBC Morphology:								
Echinocytes, n (%)	NR	0 - 2+	0 - 1+	0 - 2+	NR	0 - 3+	1+ - 2+	0 - 3+
Polychromasia (%)	NR	1 - 5	0 - 5	1 - 5	NR	1 - 5	1 - 5	3 - 9
Acanthocytes	NR	0	0	0	NR	0 - 1+	0 - Rare	0 - 2+
Anisocytosis	NR	0	0	0 - Rare	NR	0 - Rare	0 - Rare	0
Disrupted RBCs	NR	0 - 1+	0	0	NR	0 - 1+	0	0
Keratocytes	NR	0	0	0	NR	0	0 - Rare	0
WBC Comment:								
Reactive Lymphocytes	NR	Rare - 1+	0 - 2+	Rare - 2+	NR	Rare - 1+	Rare - 2+	1+ - 2+
Smudged WBCs	NR	Rare - 1+	0 - 1+	0 - 1+	NR	0 - 2+	0 - 1+	0 - Rare
Necrobiotic cells	NR	0 - Rare	0	0	NR	0	0	0
Lobulated Lymphocytes	NR	0	0	0	NR	0	0	0 - Rare

H.S.=Hypersegmented; NA = Not applicable; ND = Quantity not sufficient for test; NE=Not Evaluated; NR = No Reference range available.

¹ Reference values from Envigo website for outbred Sprague Dawley rats, 6-8 weeks old, non-fasted. ² MPC and MPM parameters are not validated and clinical interpretation is not established

Supplemental Table 2 Continued. Hematology RBC Morphology and WBC Comment Definitions

RBC Morphology

Size Variation:

Echinocytes-Evenly spiculated erythrocytes	1+ Small amount of size variation present
Acanthocytes-Unevenly spiculated erythrocytes, irregular in shape	2+ Small to moderate amount of size variation present
Anisocytosis-Variation in erythrocyte size	3+ Moderate amount of size variation present
Keratocytes-Erythrocytes with one or more projections (Blister, helmet, bite, indented)	4+ Marked amount of size variation present
Polychromasia-Erythrocytes with variable basophilia	% Observed

Shape Change (Rare-4+)

Disrupted RBCs: RBCs with smudged cell walls. Often seen on hemolyzed and/or lipemic samples.

Rare	Present but not in every oil immersion field
1+	Low numbers in every field
2+	Low to moderate numbers in every field
3+	Consistently present in a noticeable amount in each oil immersion field
4+	Unmistakably and outstandingly present in each oil immersion field

Semi-Quantitative evaluation of RBC shape change (#/high power field):

	Rare	1+	2+	3+	4+
Acanthocyte, Elliptocyte, H-J Bodies, Ovalocyte, Shistocyte, Stomatocyte	<1	1-2	3-5	6-10	>10
Eccentrocyte, Keratocyte	<1	1-4	5-9	10-15	>15
Codocyte	<1	1-5	6-9	10-25	>25
Macrocyte, Microcyte, Poikosphero, Spherocyte	<1	1-8	9-20	21-50	>50
Dacrocyte, Echinocyte	<5	5-9	10-29	30-69	>70

WBC Morphology

Rare	Present in <1% of the cells
1+	Present in a few cells, approximately 1-3%
2+	Present in few to moderate cells, approximately 3-25%
3+	Present in a moderate number of cells, approximately 25-75%
4+	Present in many cells, >75%

Hemolysis, Icterus and Lipemia

1+	Slightly
2++	Moderately
3+++	Markedly

Supplemental Table 3. Summary of Clinical Chemistry Raw Data

analyte	Males (n=11/treatment), Mean (SD)				Females (n=11/treatment), Mean (SD)			
	Reference Range ¹	T3 Vehicle	Saline Vehicle	T3	Reference Range ¹	T3 Vehicle	Saline Vehicle	T3
BUN (mg/dL)	16.1-19.5	17 (2.68)	17.09 (3.26)	16.27 (1.86)	15.9-21.7	16 (1.81)	15 (2)	13.55 (2.27)
Creat (mg/dL)	0.2	0.31 (0.03)	0.29 (0.03)	0.3 (0.06)	0.3	0.43 (0.04)	0.43 (0.06)	0.41 (0.05)
Ca (mg/dL)	11.0-11.8	10.08 (0.4)	10.05 (0.57)	9.98 (0.37)	11.2-12.0	10.1 (0.31)	10.07 (0.38)	10.11 (0.38)
Phos (mg/dL)	12.7-14.7	8.18 (0.95)	8.27 (1.08)	8.6 (0.56)	12.1-14.3	7.34 (0.72)	6.75 (1.14)	7.41 (0.73)
Mg (mg/dL)	NR	2.3 (0.13)	2.25 (0.14)	2.38 (0.2)	NR	2.52 (0.24)	2.36 (0.23)	2.55 (0.15)
TP (g/dL)	6.0-6.6	6.11 (0.16)	6.05 (0.23)	5.81 (0.13)	6.4-7.0	6.41 (0.31)	6.47 (0.3)	6.05 (0.21)
Alb (g/dL)	2.9-3.3	3.22 (0.1)	3.24 (0.13)	3.18 (0.07)	3.2-4.0	3.48 (0.16)	3.49 (0.17)	3.21 (0.12)
Glob (g/dL, calc.)	3.1-3.3	2.89 (0.13)	2.81 (0.12)	2.63 (0.12)	2.9-3.3	2.93 (0.2)	2.98 (0.15)	2.85 (0.13)
Na (mmol/L)	146.1-150.1	142.08 (1.26)	142.55 (1.37)	142.18 (1.19)	144.9-148.7	142.09 (1.78)	142.91 (1.08)	141.64 (1.77)
Cl (mmol/L)	103.8-108.0	100.42 (1.5)	100.64 (1.15)	99.55 (1.23)	104.1-110.1	100.18 (1.34)	100.82 (2.08)	100.45 (1.78)
K (mmol/L)	5.3-7.9	5.08 (0.32)	5.02 (0.26)	4.95 (0.34)	5.6-8.2	4.56 (0.2)	4.43 (0.22)	4.52 (0.32)
HCO ₃ (mmol/L)	NR	21.59 (2.08)	21.79 (1.79)	22.24 (1.54)	NR	19.27 (2.61)	19.37 (2.11)	19.8 (2.9)
Osmol (calc.)	NR	287.75 (1.88)	288.45 (2.68)	287.82 (1.99)	NR	286.64 (2.99)	287.64 (2.53)	286.09 (3.45)
An Gap (calc.)	NR	25.25 (2.83)	25.18 (2.79)	25.45 (2.19)	NR	27.27 (3.79)	27.27 (3.33)	25.91 (3.78)
T. Bili (mg/dL)	0.1	0.13 (0.04)	0.11 (0.03)	0.11 (0.03)	0.0-0.6	0.16 (0.05)	0.14 (0.05)	0.17 (0.04)
ALP (U/L)	277.4-341.0	208 (43.83)	207.82 (41.31)	203.64 (38.46)	184.9-234.3	126.45 (45.34)	135.91 (21.16)	112.18 (14.9)
GGT (U/L)	NR	<3 (0)	<3 (0)	<3 (0)	NR	<3 (0)	<3 (0)	<3 (0)
ALT (U/L)	58.0-78.0	52.25 (15.06)	47.45 (10.71)	46.45 (9.34)	48.2-92.0	49.18 (16.14)	45.73 (24.76)	43.82 (6.12)
AST (U/L)	55.0-180.6	95.17 (52.79)	77.73 (22.35)	75.91 (17.98)	51.8-188.8	91.45 (69.28)	75.64 (36.74)	99.91 (42.83)
CK (U/L)	NR	264.92 (152.13)	255.64 (112.18)	230.36 (68.03)	NR	164.82 (34.94)	145 (59.84)	179.82 (49.46)
Gluc (mg/dL)	NR	150.33 (22.6)	147.18 (17.65)	154.45 (16.56)	NR	137.64 (13.23)	137 (12.53)	157.36 (9.66)
Chol (mg/dL)	115.8-132.8	113.75 (11.35)	115.64 (15.99)	110.55 (11.41)	111.3-135.9	101.09 (9.47)	92.73 (9.14)	105.91 (15.36)
Amylase (U/L)	NR	657.17 (38.62)	662.91 (66.43)	661.09 (57.99)	NR	535.82 (52.19)	561.18 (59.33)	550.73 (59.11)
LIH [^]	NR	NA (NA)	NA (NA)	NA (NA)	NR	NA (NA)	0 0 0 (0 0 0)	NA (NA)

NA = Not applicable; ND = Quantity not sufficient for test; NE=Not Evaluated; NR = No Reference range available;

¹ Reference values from Envigo website for outbred Sprague Dawley rats, 6-8 weeks old, non-fasted

Supplemental Table 3 Continued. Explanation of Clinical Chemistry Chromatic Substance

Approximate Concentration of Chromatic Substance[^]

Flag	LIP	ICT (mg/dL Bilirubin)	HEM (mg/dL Hemoglobin)
No	<0.0130	< 0.0867	< 0.0548
+	0.013	0.0867	0.0548
++	0.02	0.2099	0.1784
+++	0.0318	0.4554	0.3369
++++	0.0433	0.5009	0.8537
+++++	0.0613	0.6231	1.6909

L = Lipemia

I = Icterus

H = Hemolysis

Grading range from 0 (lowest) to 6 (highest).

Lipemia of 1-5+ may affect Lipase

Lipemia of 4-5+ may affect Glucose

Lipemia of 5+ may affect BUN, Crea, Ca, Phos, Mg, TP, Alb, HCO₃, Tbil, ALP, GGT, ALT, AST, Chol and Amy

Icterus of 2-5+ may affect Cholesterol

Icterus of 3-5+ may affect CK, Lipase and BUN

Icterus of 4-5+ may affect ALP, ALT, Glu, Mg and TP

Hemolysis of 1-5+ may affect AST

Hemolysis of 2-5+ may affect CK and Mg

Hemolysis of 3-5+ may affect Amyl and TP

Hemolysis of 4-5+ may affect Chol, Glu and Phos

Any Lipemia, Icterus, or Hemolysis of 6+ may affect all test results.

Supplemental Table 4. Summary of Histopathology Data for Pulmonary Tissues

		INCIDENCE OF FINDINGS					
		5 Days					
		MALE			FEMALE		
Group		1	2	3	1	2	3
Group Description		T3 Control	Saline Control	T3	T3 Control	Saline Control	T3
Number of Animals		12	11	11	11	11	11
Number Examined		12	11	11	11	11	11
Tissue	Findings and Scores						
Lung Left Lobe	<i>Number of Animals Examined</i>	12	11	11	11	11	11
	<i>Normal</i>	5	7	7	7	8	6
	Alveolar macrophage focus	1	0	0	0	0	0
	<i>...score 1*</i>	1	0	0	0	0	0
	Inflammation, chronic, bronchioalveolar	5	4	3	2	2	4
	<i>score 1</i>	5	4	3	2	2	4
	Alveolar histiocytosis	0	1	1	2	1	1
	<i>score 1</i>	0	1	1	2	1	1
	Congestion, acute	1	0	0	0	0	0
	<i>score 1</i>	0	0	0	0	0	0
<i>score 2</i>	1	0	0	0	0	0	
Autolysis	1	0	0	0	0	0	
<i>score 1</i>	0	0	0	0	0	0	
<i>score 2</i>	1	0	0	0	0	0	
Lung Right Cranial and Caudal Lobe, Middle and Accessory Lobes	<i>Number of Animals Examined</i>	12	11	11	11	11	11
	<i>Normal</i>	5	6	4	7	6	3
	Inflammation, chronic, bronchioalveolar	5	3	6	4	3	8
	<i>score 1</i>	5	3	6	4	3	8
	Alveolar histiocytosis	1	2	1	0	2	3
	<i>score 1</i>	1	2	1	0	2	3
	Congestion, acute	1	0	0	0	0	0
	<i>score 1</i>	0	0	0	0	0	0
	<i>score 2</i>	1	0	0	0	0	0
	Autolysis	1	0	0	0	0	0
<i>score 1</i>	0	0	0	0	0	0	
<i>score 2</i>	1	0	0	0	0	0	
Tracheobronchial Lymph Nodes		-	-	-	-	-	-
Trachea, including the bifurcation of the major bronchioles.		-	-	-	-	-	-

*Scoring: - Tissue normal, no finding present; 1 Present, but minimal feature; 2 Notable feature, mild; 3 Prominent feature that does not disrupt tissue architecture, and is not overwhelming; 4 Overwhelming feature, or feature that effaces or disrupts tissue architecture, severe.