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Preclinical Pharmacology and Pharmacokinetics of Inhaled Hexadecyl-Treprostinil (C16TR), a Pulmonary Vasodilator Prodrug

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Abbreviations:

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AUC	Area under the plasma TRE concentration curve
BLQ	Below the Limit of Quantitation
C10TR	Decyl-treprostinil
C12TR	Dodecyl-treprostinil
C14TR	Tetradecyl-treprostinil

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C16TR	Hexadecyl-treprostinil
C16OTR	Ether-linked hexadecyl-treprostinil derivative
C_{max}	Mean maximum plasma concentration
CHO-K1	Chinese hamster ovary K1 cell line
CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DP_1	Prostaglandin D_2 receptor 1
DSPE-PEG2000	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000
EP_1	Prostaglandin E_2 receptor 1
EP_2	Prostaglandin E_2 receptor 2
EP_3	Prostaglandin E_2 receptor 3
EP_4	Prostaglandin E_2 receptor 4
FP	Prostaglanding F2α receptor
HPLC/MS/MS	High performance liquid chromatography/mass spectrometry
HPLC/UV	High performance liquid chromatography/ultraviolet
HR	Heart rate
IBD	Immediately before dose
IC50	Half maximum inhibitory concentration
INS1009	Hexadecyl-treprostinil formulated in a lipid nanoparticle
IP	Prostaglandin I_2 receptor
IV	Intravenous
K_2-EDTA	Di-potassium ethylenediaminetetraacetic acid
LLOQ	Lower limit of quantitation
LNP	Lipid nanoparticle
MW	Molecular Weight
NA	Not applicable
NT	Not tested
PAH	Pulmonary arterial hypertension

PAP	Pulmonary arterial pressure
PBS	Phosphate buffered saline
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF2_\alpha$	Prostaglandin $F2_\alpha$
PK	Pharmacokinetics
PPP	Platelet poor plasma
PRP	Platelet rich plasma
SaO_2	Arterial oxygen saturation
SAP	Systemic arterial blood pressure
SD	Standard deviation
SEM	Standard error of the mean
T_{max}	Time to maximum plasma concentration
TRE	Treprostinil

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ABSTRACT

This report describes the preclinical pharmacology and pharmacokinetics (PK) of hexadecyl-treprostinil (C16TR), a prodrug of treprostinil (TRE), formulated in a lipid nanoparticle (LNP) for inhalation as a pulmonary vasodilator. C16TR showed no activity (> 10 μM) in receptor binding and enzyme inhibition assays including binding to EP_2, DP_1, IP and EP_4; TRE potently bound to each of these prostanoid receptors. C16TR had no effect (up to 200 nM) on platelet aggregation induced by adenosine diphosphate (ADP) in rat blood. In hypoxia-challenged rats, inhaled C16TR-LNP produced dose-dependent (0.06 - 6 μg/kg) and sustained pulmonary vasodilation over 3 h; inhaled TRE (6 μg/kg) was active at earlier times but lost its effect by 3 h. Single and multiple dose PK studies of inhaled C16TR-LNP in rats showed proportionate dose-dependent increases in TRE C_{max} and AUC for both plasma and lung; similar results were observed for dog plasma levels in single dose PK studies. In both species, inhaled C16TR-LNP yielded prolonged plasma TRE levels and a lower plasma TRE C_{max} compared to inhaled TRE. Inhaled C16TR-LNP was well tolerated in rats and dogs with TRE-related side effects such as cough, respiratory tract irritation and emesis only seen after high inhaled doses of C16TR-LNP in dogs. In guinea pigs, inhaled TRE (30 μg/mL) consistently produced cough but C16TR-LNP (30 μg/mL) elicited no effect. These results demonstrate that C16TR-LNP provides long acting pulmonary vasodilation, is well tolerated in animal studies and may necessitate less frequent dosing than inhaled TRE with possibly fewer side effects.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a life-threatening and progressive disease that is characterized by constriction and remodeling of the pulmonary vasculature leading to increased pulmonary vascular resistance and pulmonary arterial pressure (PAP), most often resulting in right heart failure (Stamm et al., 2011; Frumkin, 2012). Current therapies to treat PAH have been directed towards reversing the pulmonary vasoconstriction and more recently to the resolution of pulmonary vascular remodeling which includes smooth muscle cell proliferation and partial or complete occlusion of pulmonary blood vessels. Therapies currently used for the treatment of PAH include prostanoids (Skoro-Sajer et al., 2007; Nadler and Edelman, 2010; Channick et al., 2012**)**, endothelin receptor antagonists (Abman, 2009), phosphodiesterase type 5 inhibitors (Wilkins et al., 2008), a soluble guanylate cyclase activator (Khaybullina et al., 2014) and calcium channel blockers (Taichman et al. 2014).

Treprostinil (TRE) is a relatively stable analogue of prostacyclin that has a longer plasma half-life than early prostacyclin agonists such as Iloprost (Ventavis®) and epoprostenol (Flolan/Veletri®). TRE has therapeutic benefit when given by continuous infusion (Remodulin®), oral (Orenitram®) and inhaled (Tyvaso®) routes of administration. However, Remodulin administration is associated with significant injection site pain (subcutaneous) or concern of infection (intravenous) (Simonneau et al., 2002; Remodulin, 2014) while Orenitram and Tyvaso require multiple doses daily and efficacy may not be fully maintained over a 24 h period (Channick et al., 2012; Tyvaso® 2013; Orenitram® 2014; Feldman et al. 2015). Inhaled TRE is also associated with a number of adverse side effects which limits the dose. For example, cough, headache, laryngeal irritation, emesis, flushing, nausea and hypotension are the side effects most frequently found with Tyvaso which is taken 4-times daily (Voswinckel et al., 2006; Channick et al., 2012; Nadler and Edelman, 2010; Tyvaso®, 2013).

In sheep, Sandifer et al. (2005) demonstrated that continuously inhaled TRE showed greater efficacy with fewer systemic side effects than continuously infused drug. However, in that study inhaled TRE had to be administered continuously in order to maintain a consistent effect; this of course is impractical for patients. We reasoned that an inhaled slow-release formulation of drug might achieve long-acting pulmonary vasodilation and elicit fewer systemic side effects. To accomplish this, a series of ester-linked prodrugs of TRE formulated in a lipid nanoparticle (LNP) and optimized for delivery by inhalation were developed (Leifer et al., 2017). The culmination of this research identified hexadecyl-treprostinil (C16TR) as a lead inhalation prodrug candidate.

In this report, we describe the preclinical pharmacology and pharmacokinetic (PK) properties of the C16TR prodrug formulated in a LNP for inhalation. Some of these results have appeared in abstracts and posters at the American Thoracic Society and European Respiratory Society Conferences (Leifer et al., 2014; Malinin et al., 2014, 2015, 2017; Chapman et al., 2015, 2017; Li et al., 2016). The experiments performed herein describe: 1) *in vitro* assays on human prostanoid receptor binding, 2) platelet aggregation evaluations in rat blood, 3) *in vivo* activity in a rat model of acute hypoxia-induced pulmonary hypertension, 4) PK in rats and dogs and 5) assessment on the cough reflex in guinea pigs.

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MATERIALS and METHODS

Materials. TRE was obtained from Chirogate International (Taoyuan County, Taiwan, Republic of China). Dodecanol, tetradecanol, hexadecanol, 1,4 dioxane, amberlyst-15 resin, and squalane were acquired from Sigma (St. Louis, MO, USA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000, ammonium salt) and Cholesterol-PEG2000 were obtained from NOF America (White Plains, NY, USA). Dodecyl-TRE (C12TR), tetradecyl-TRE (C14TR), and C16TR were synthesized at Insmed Incorporated (Bridgewater, NJ, USA) via esterification of TRE acid in the presence of long chain alcohols catalyzed by the acidic resin Amberlyst-15 as described previously (Leifer et al., 2017). Phosphate buffered saline (PBS) was acquired from Mediatech (Manassas, VA, USA).

LNP Prodrug formulations. Two different LNP prodrug formulations of C16TR were used. One formulation contains C16TR and the excipients squalane and DSPE-PEG2000 at a molar ratio of 45/45/10, suspended in PBS. This formulation has previously been termed INS1009 (Chapman et al., 2015, 2017; Han et al., 2016 a, b; Malinin et al., 2015, 2017; Li et al., 2016). The other formulation contains C16TR and squalane, but with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol-PEG2000; the molar ratios of these 4 ingredients were 40/40/10/10. This formulation has been previously termed T623 (Leifer et al., 2014, 2017; Malinin et al., 2014). Two additional TRE prodrugs were evaluated in dogs and consisted of C14TR or C12TR that were formulated in LNP containing squalane, DOPC and cholesterol-PEG2000 in the ratio of 40/40/10/10. Nanoparticles were suspended in PBS and exhibited mean diameters ranging from 100 to 150 nm as measured by dynamic light scattering. An ether-linked derivative of hexadecyl-treprostinil (C16OTR) was also used that incorporates an ether bond linking the TRE moiety to the hexadecyl group; this compound was formulated in LNPs as C16OTR/squalane/DSPE-PEG2000 in a molar ratio of 45/45/10. The chemical structures of C16TR and C16OTR are shown in Figure 1.

Radioligand Binding Assays. Radioligand binding assays for human Prostaglandin D_2 receptor 1 (DP_1), Prostaglandin I_2 receptor (IP), Prostaglandin E_2

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receptor 1 (EP_1), Prostaglandin E_2 receptor 2 (EP_2), Prostaglandin E_2 receptor 3 (EP_3), Prostaglandin E_2 receptor 4 (EP_4), Prostaglandin F2α receptor (FP) and Chemoattractant receptor-homologous molecule expressed on Th2 cells ($CRTH_2$) receptors were performed by Eurofins Panlabs (Taipei, Taiwan, Republic of China). The following radioligands were used: [^{3}H] Prostaglandin D_2 (PGD_2) (DP_1, $CRTH_2$), [^{3}H] Prostaglandin E_2 (PGE_2) (EP_1, EP_2, EP_3, EP_4), [^{3}H] Prostaglandin $F2_\alpha$ ($PGF2_\alpha$) (FP) and [^{3}H] Iloprost (IP). The compounds were incubated at 25 ˚C for either 1 h (EP_1, FP and IP) or 2 h (DP_1, $CRTH_2$, EP_2, EP_3 and EP_4) with human recombinant Chinese hamster ovary K1 cell line (CHO-K1 cells) expressing the appropriate human prostanoid receptor. Binding activity was measured as the inhibition (%) of radioligand binding and half maximum inhibitory concentration (IC_{50}) values were determined by a non-linear, least squares regression analysis using MathIQ™ (ID Business Solutions Ltd, Guildford, UK). Because C16TR itself is insoluble in water, a micelle formulation containing C16TR and DSPE-PEG2000 in a 70/30 molar ratio was used to facilitate availability of the prodrug in this assay.

Platelet aggregation. Blood samples from male Sprague Dawley rats (Charles River Laboratories, Quebec, Canada) were centrifuged to extract platelet rich plasma (PRP) and platelet poor plasma (PPP). PRP was diluted with PPP to yield samples containing 3.5 X 10^8 platelets/mL. Platelet aggregation was induced by the addition of adenosine diphosphate (ADP) (10 µM final concentration) measured with a Chrono-log aggregometer. Test concentrations for C16TR formulated in squalane/DOPC/cholesterol-PEG2000 lipid nanoparticles were 50, 100, 200, and 400 nM (TRE equivalents). Values for platelet aggregation in the presence of C16TR formulated LNP, the LNP vehicle alone and TRE were normalized to values obtained in the presence of PBS and the results were expressed as the % inhibition due to drug or control.

Hypoxia Challenge in Rats. Male Sprague Dawley rats (Charles River Laboratories, Quebec, Canada) ranging in weight from 300-350 g were anesthetized with a 3% isoflurane-oxygen and then transitioned to intravenous (i.v.) ketamine/xylazine (mixture of 10 and 1 mg/mL, respectively at an infusion rate of 0.02 mL/min). The rats were prepared with tracheal, pulmonary arterial and arterial blood pressure catheters and systemic arterial blood pressure (SAP), heart rate (HR) and PAP were measured throughout the study. A pulse oximeter was placed on the paw to measure arterial oxygen

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saturation (SaO_2). The rats were artificially ventilated and drugs were delivered using an Aeroneb® Pro nebulizer (Aerogen, Galway, Ireland) that was interposed into the inspiratory line of the ventilator. The volume of test article nebulized was fixed at 300 µL and doses were varied by altering the drug concentration.

Following the measurement of PAP, SAP, HR and SaO_2 during ventilation on room air (21% O_2), the inspired gas was switched to a hypoxic gas mixture (10% O_2) and maintained at hypoxic levels ($SaO_2 \approx$ 45-60%) for the duration of the study. Once a 5 min stable elevation in PAP had been achieved during the hypoxia exposure, physiological parameters, including PAP, were measured to represent their hypoxic baseline values. The following test articles [PBS, TRE, C16TR formulated in squalane/DSPE-PEG2000 LNP (0.06-6 µg/kg) and C16OTR formulated in the same LNP formulation to C16TR (6 µg/kg)] were nebulized, delivered directly into the tracheal tube and physiological parameters were measured over a 180 min period. The data were sampled over 20 s periods (200 data points per second) and averaged as a single value at times of 0-5, 5.3-20, 20.3-60, 60.3-100, 100.3-147 and 147.7-180 min after nebulization of the drugs. The changes induced by the test articles were measured at each of these time intervals and expressed as a % reduction of the hypoxic baseline value. A single dose of test article was given per rat. In studies involving inhaled C16TR-LNP (6 µg/kg) and TRE (6 µg/kg), blood samples (0.2 mL/sample) were collected at times of 3, 20, 40, 60, 90 and 180 min after drugs and lungs were harvested at the end of the studies, i.e. 180 min. The concentration of TRE and C16TR in the plasma and lung were measured by high performance liquid chromatography/mass spectrometry (HPLC/MS/MS). Statistical significance differences between the treatment groups were determined using a one way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test.

Pharmacokinetics in Rats. Male Sprague Dawley rats (Charles River Laboratories, Quebec, Canada) ranging in weight from 300-350 g were placed in a 12-port rodent nose-only inhalation tower (CH Technologies, Westwood, NJ, USA) for the exposure to inhaled drugs. The total airflow through the tower was maintained at 6 L/min and a glass filter was connected to one of the exposure ports to measure the aerosol drug concentration. Drug samples were collected on the filter for 5 min at a vacuum flow of

0.5 L/min and measured by high performance liquid chromatography/ultraviolet (HPLC-UV). The inhaled pulmonary dose (µg/kg) of C16TR was derived from the relationship of aerosol drug concentration in the nose-only inhalation tower, the duration of exposure to the test articles, minute ventilation based upon body weight and a 10% deposition factor as described previously (Alexander et al., 2008; Wolff and Dorato, 1993). Values for C16TR were converted (0.635) to molar equivalents of TRE based upon the molecular weights (MW) of C16TR (MW = 614.9) and TRE (MW = 390.5).

A single dose PK study was performed with C16TR formulated in squalane, DOPC, cholesterol-PEG2000 (40/40/10/10). Evaluations were performed with inhaled pulmonary doses of 0.6, 1.8, 6 and 18 µg/kg. Blood and lung tissue samples were obtained in 3 rats/time point at each time point of 1, 2, 3, 6, 12, 20 and 24 h after the start of the drug exposure. A repeat dose PK study was also performed using C16TR formulated in squalane, DSPE-PEG2000. In this experiment, cohorts of 4 rats/dose were exposed for 1, 7 or 14 consecutive days and evaluations were performed with inhaled pulmonary doses of 0.6, 1.8, 6 and 18 µg/kg. Blood samples were obtained from each rat at times of 1, 3, 6 and 24 h after the start of the drug exposure and lungs were harvested at the end of the study i.e. 24 h after the final drug dose. For all PK studies, the blood samples were placed on ice until centrifugation to extract the plasma. Both the plasma and lung samples were stored at -50 ºC prior to analysis of their TRE and C16TR concentrations using HPLC/MS/MS.

The following calculations were performed on the plasma TRE data: mean maximum plasma concentration (C_{max}, ng/ml), the time to maximum plasma concentration (T_{max}, min) and area under the plasma TRE concentration curve over a 24 h period ($AUC_{0\text{-}24h}$ ng*h/ml) measured by the trapezoidal method (Chow and Liu, 2007). For the lung tissue, the concentration of both C16TR and TRE was measured in lung homogenate and converted to the total equivalent concentration of TRE. Results were expressed as the TRE equivalent content per gram of lung tissue (ng/g).

Pharmacokinetics in Dogs. Experiments were performed on 6 male and 6 female beagle dogs (Marshall BioResources, North Rose, NY, USA). The dogs were

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anesthetized with propofol (7 mg/kg, i.v.), intubated with a cuffed endotracheal tube and mechanically ventilated on room air at a tidal volume of 10 mL/kg and 15 breaths/min. A dosimetry system similar to that previously described for use in dogs (Kuehl et al., 2010) was used to deliver inhaled TRE and the LNP formulations (squalane, DOPC, cholesterol-PEG2000) of C12TR, C14TR and C16TR. The compounds were nebulized with an Aeroneb Lab nebulizer (Aerogen, Galway, Ireland) and delivered directly into a 500mL expansion chamber on the inspiratory limb of the ventilator. The power interrupter on the nebulizer was set to 1s on and 2 s off to minimize condensation of nebulized material on the inner walls of the system. The concentration of inhaled drug was measured by collecting drug deposited on a filter attached to the end of the endotracheal tube per unit of time. Inhaled drug dose (µg/kg) was measured from the relationships of aerosol drug concentration, the duration of drug exposure and minute ventilation based upon body weight as described previously (Alexander et al., 2008).

At the end of the drug delivery, the anesthesia infusion was discontinued and the dogs were weaned off the ventilator. After full recovery from the anesthesia, which took only a few minutes, the dogs were returned to their home cages. The dogs were checked for clinical signs for up to 3 days after dosing with the drugs. Blood collections were performed at times of 0.02, 0.08, 0.16, 0.50, 1, 2, 4, 8, 12, 16, 20, 24, 48 and 72 h after drug administration with approximately 2 mL/sample of blood taken at each time point (28 mL blood total). The blood samples were collected into tubes containing di-potassium ethylenediaminetetraacetic acid (K_2-EDTA) anticoagulant and put immediately on ice until processed. Processing occurred within 2 h of blood collection. The samples were centrifuged (for 10 min at approximately 2000g, at 2 to 8 ºC) to obtain plasma. Plasma (approximately 500 µL for each aliquot) was stored frozen at nominal -80 ºC (± 10 ºC) and the concentration of TRE was measured using a qualified HPLC/MS/MS method. The plasma TRE samples were used to determine C_{max}, T_{max}, AUC $_{0\text{-}24h/0\text{-}72h}$.

Measurement of TRE and C16TR in blood plasma and lung homogenate. TRE and C16TR were measured in plasma and lung homogenate samples by HPLC/MS/MS assays. The TRE assay had a lower limit of quantitation (LLOQ) of 25 pg/mL for TRE in

plasma and 250 pg/mL in lung homogenate. The C16TR assay had a LLOQ of 300 pg/mL in lung homogenate.

TRE and C16TR were extracted from plasma and lung homogenate samples by liquid extraction in acetonitrile/water/acetic acid (50/50/0.5, v/v/v). 1-Naphthoxyacetic acid was used as an internal standard for TRE and decyl-TRE (C10TR) or C14TR-TRE were used as internal standards for C16TR. Extracts were analyzed using Ace 3 C18 (Kinetex Phenyl-Hexyl) reverse phase analytical column at a flow rate of 1 mL/min with an injection volume of 20 µL. The gradient method was used with Mobile Phase A prepared as 1% formic acid in water and Mobile Phase B as 100% Acetonitrile. The starting concentration of Mobile Phase B in a gradient was 35%. Detection was done by HPLC/MS/MS (AB SCIEX, Framingham, MA, USA).

Cough Reflex in Guinea Pigs. Cough was measured in conscious, unrestrained guinea pigs (250-350 g) (Charles River Laboratories Inc., Wilmington, MA, USA) by three established criteria (Morice et al., 2007): changes in ventilation using a whole body plethysmograph, cough sounds measured with a microphone and visual inspection of the postural changes during cough. The guinea pigs were exposed to nebulized PBS (n=6), TRE at nebulizer concentrations of 1 µg/mL (n = 1), 3.3 µg/mL (n = 3), 10 µg/mL (n = 4), 30 µg/mL (n = 7) and C16TR-LNP at a concentration of 30 µg/mL (TRE equivalent) (n = 6). A guinea pig exposed to TRE at 300 µg/mL experienced adverse effects and dosing was discontinued after 4 min. The compounds were given directly into the whole body plethysmograph using an Ultra-Neb Pro nebulizer (nebulizer output of 0.36 mL/min) for 10 min and the total number of coughs were measured during the 10 min of nebulization and for an additional 20 min period after the test article was given. Airflow through the plethysmograph was maintained at a constant flow rate of 2 L/min and was derived from a compressed air source. Statistical significance between the treatment groups was determined using Kruskal-Wallis analysis of variance (non-parametric) followed by the Dunn method for joint ranking (Kruskal and Wallis, 1952; Dunn, 1961).

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Animal Care and Use. The experimental protocols involving animals were approved by the Institutional Animal Care and Use Committees at the following institutions: IPS Therapeutique Inc., Sherbrooke, Quebec, Canada; Lovelace Respiratory Research Institute, Albuquerque, NM, USA and Envigo CRS Inc., East Millstone, NJ, USA. These Institutions are facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

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RESULTS

Binding to Prostanoid Receptors. TRE had binding to the following prostanoid receptors with an order of potency (estimated IC_{50}) of: EP_2 (0.014 μM) > DP_1 (0.023 μM) > IP (0.079 μM) > EP_1 (0.20 μM) > EP_3 (0.25 μM) > EP_4 (0.92 μM); TRE had no activity (> 10 μM) against $CRTH_2$ and FP (Table 1). In contrast, C16TR had no binding (> 10 μM) to EP_2, DP_1, IP and EP_4 receptors; evaluations were not performed against the other prostanoid receptors. Similar results were found with C16OTR that had no binding (> 10 μM) to these prostanoid receptors.

Platelet aggregation. TRE inhibited platelet aggregation in a concentration-dependent manner at ≥ 50 nM with a mean maximum effect of 64%. C16TR formulated in a LNP was inactive at 200 nM with only 13.5% inhibition at 400 nM (Figure 2). The LNP vehicle minus C16TR had no effect (data not shown).

Hypoxia Challenge in Rats. Following challenge with hypoxia, PAP increased from values of 17.8 ± 0.6 mmHg during normoxia to 23.2 ± 0.8 mmHg during hypoxia (n = 32). This elevation in PAP did not appreciably change during the 180 min of hypoxia exposure and was unaffected by exposure to nebulized PBS (Figure 3). Exposure of hypoxia-challenged rats to C16TR-LNP dose-dependently (0.6-6 μg/kg) reduced PAP; a lower dose of 0.06 μg/kg was inactive (n = 3). At the highest dose of C16TR-LNP (6 μg/kg), the PAP was reduced to 13 ± 1 mmHg that is slightly below the normoxic levels. Dose-response studies with inhaled TRE (0.006 - 6 μg/kg) in hypoxic rats also reduced PAP (data not shown) with maximum effects observed at a dose of 6 μg/kg (Figure 4). A higher dose of TRE (10 μg/kg, n = 4) produced no further reduction in PAP (Normoxia PAP = 18.6 ± 1.6 mmHg; Hypoxia PAP = 25.1 ± 2.6 mmHg; Hypoxia + TRE PAP = 18.0 ± 1.8 mmHg). There were no changes in SAP and HR with any treatments (data not shown).

There were two important differences in the pulmonary vasodilator responses between inhaled C16TR-LNP and inhaled TRE (Figure 4). First, a reduction in PAP occurred during the nebulization with inhaled TRE (6 μg/kg) and was already 15 % below

the baseline value by 5 min after challenge whereas the reduction in PAP for inhaled C16TR-LNP (6 μg/kg) was delayed and not observed until 5-20 min after administration (Figure 4). Second, over 180 min, the reduction in PAP with inhaled TRE (6 μg/kg) trended back to the hypoxic baseline values whereas a sustained and significant reduction in PAP (relative to TRE) was seen with inhaled C16TR-LNP (6 μg/kg) (Figure 4). Inhaled LNP formulated C16OTR (6 μg/kg), the ether-linked C16TR compound, had no effect. PAP measurements could not be reliably obtained beyond 180 min in this model of hypoxia-challenged rats.

Plasma concentrations of TRE were highest (3.44 ng/mL) by 3 min for inhaled TRE (6 μg/kg) (Table 2). In contrast, the plasma TRE level for inhaled C16TR-LNP (6 μg/kg) was only 0.06 ng/mL at 3 min and slowly increased to the highest value of 0.22 ng/mL at 180 min (Table 2). In the lung, the TRE-equivalent concentration (C16TR+TRE, see Methods) was 67-fold higher for inhaled C16TR-LNP (438 ng/g lung tissue) compared to inhaled TRE (6.5 ng/g lung tissue). No TRE was detected in the plasma or lungs for inhaled C16OTR.

Pharmacokinetics in Rats. Single doses of inhaled LNP formulated C16TR (0.6-18 μg/kg) were administered and the concentration of TRE in the plasma increased proportionately in a dose-dependent manner (Figure 5A). The elimination of TRE from the plasma followed a first-order exponential decline. There were dose-dependent (0.6-18 μg/kg) increases in both plasma TRE C_{max} and AUC $_{0\text{-}24h}$ for inhaled C16TR-LNP with a plasma TRE T_{max} at 1 h at all doses (Table 3). The concentration of TRE and C16TR also increased in the lungs with increasing inhaled doses of C16TR-LNP (Figure 5B) with the majority of the combined TRE + C16TR (TRE equivalent concentration) present as C16TR. Compared to the TRE concentration in the plasma, the lung TRE-equivalent concentration was approximately 1,000-times higher.

Inhaled C16TR-LNP (0.6-18 μg/kg) that had been dosed daily for 14 consecutive days was well tolerated in rats and associated with dose proportional increases in both plasma TRE C_{max} and AUC $_{0\text{-}24h}$ (Table 4). There was no evidence of plasma TRE accumulation after 14 days of dosing with inhaled C16TR-LNP although there were some fluctuations of C_{max} and AUC $_{0\text{-}24h}$ values over time at the 0.6 and 6 μg/kg doses; however,

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the reason for this is unclear because these parameters generally remained steady over time at the other doses (Table 4). The lung concentration of TRE following repeat dosing with inhaled C16TR-LNP did not show any substantive variation over time and there was no evidence of TRE accumulation with repeat dosing (Figure 6).

Pharmacokinetics in Dogs. There was a marked difference in the plasma TRE PK profile between inhaled TRE and the inhaled LNP TRE-prodrugs (C16TR, C14TR and C12TR) (Figure 7). The highest plasma TRE was seen within a few minutes after inhalation of TRE and the TRE prodrugs and at a targeted inhaled dose of 18 μg/kg, the plasma TRE C_{max} was highest with inhaled TRE (5.5 ng/mL), slightly lower with inhaled C12TR-LNP (1.2 ng/mL) and 14-times lower with inhaled C14TR-LNP and C16TR-LNP (0.4 ng/mL) (Table 5). Furthermore, TRE was not detected in the plasma by 24 h after inhaled TRE, but was present at this time after inhalation with each of the TRE-prodrugs (Figure 7). The rank order values for $AUC_{0\text{-}24h}$ at a targeted inhaled dose of 18 μg/kg was highest for inhaled TRE (5.08 ng*h/mL) and lower with inhaled C12TR-LNP (4.20 ng*h/mL), inhaled C14TR-LNP (2.91 ng*h/mL) and inhaled C16TR-LNP (2.08 ng*h/mL) (Table 5).

Inhaled C16TR-LNP produced dose-dependent increases in plasma TRE with levels of TRE detected up to 72 h after inhalation for doses of 46 and 95 μg/kg (Figure 8A). Corresponding values for plasma TRE C_{max} and $AUC_{0\text{-}24h/0\text{-}72h}$ also increased in a dose-dependent manner (Table 5). In contrast, plasma concentrations of TRE were highest immediately after inhalation of TRE (5 and 16 μg/kg) but were below the limit of quantitation (BLQ) by 24 h (Figure 8B).

Inhaled TRE produced cough, rapid shallow breathing, emesis and pale gums at a delivered pulmonary dose of 16 μg/kg. Similar findings were observed with inhaled C12TR-LNP at a delivered pulmonary dose of 19 μg/kg but not with inhaled C16TR-LNP and C14TR-LNP until higher delivered pulmonary doses (93-95 μg/kg) were given (Table 5).

Cough Reflex in Guinea Pigs. A preliminary evaluation with inhaled TRE demonstrated that a 30 μg/mL concentration could consistently evoke coughs in all

guinea pigs (7 of 7) tested. Lower concentrations of TRE (1, 3.3, and 10 μg/mL) did not consistently cause cough in each guinea pig and were therefore not deemed appropriate to compare the effect of inhaled C16TR-LNP. An additional guinea pig was administered TRE at 300 μg/mL but the exposure was discontinued due to adverse effects. PBS was also administered to 6 guinea pigs and did not induce cough.

The cough response to inhaled TRE was characterized by bouts of high frequency cough with relatively low cough sounds. At a concentration of 30 μg/mL, TRE consistently produced cough in all guinea pigs (mean = 36 ± 9 coughs; range = 17 to 82 coughs); coughing occurred with between 1-4 bouts per guinea pig. In contrast, inhaled C16TR-LNP at a concentration of 47 μg/mL (C16TR molar concentration equivalent to 30 μg/mL of TRE) did not induce cough in any guinea pig (Figure 9).

DISCUSSION

Inhaled TRE is a pulmonary vasodilator used for the treatment of PAH. Although this compound has achieved some clinical success (Channick et al., 2012), it may not have reached its full potential due to its relatively short duration of action and the number of adverse side effects it evokes (Voswinckel et al., 2006; Channick et al., 2012; Nadler and Edelman, 2010). To improve upon the duration of action and reduce side effects of TRE, LNP formulations of C16TR were developed resulting in a lead compound, INS1009 (Leifer et al., 2017). In this report, pharmacological and pharmacokinetic studies were performed to highlight some of the important features of inhaled C16TR-LNP. We found that C16TR possessed no inherent pharmacological activity on prostanoid receptors and was weakly active in a rat platelet aggregation assay. Inhaled LNP formulations of C16TR produced long acting pulmonary vasodilation in hypoxia-challenged rats most likely due to the slow conversion of TRE from C16TR present in the lungs. Lower plasma TRE C_{max} was found with inhaled C16TR-LNP compared to inhaled TRE in rats and dogs and there was no evidence of drug accumulation in the plasma and lungs with repeat dosing for 14 consecutive days in rats. Inhaled C16TR-LNP was well tolerated in rats, guinea pigs and dogs with a lower incidence of TRE-related side effects, such as cough, compared to inhaled TRE.

To establish whether C16TR can be classified as an inactive prodrug, evaluations were performed in receptor binding and enzyme inhibition assays. This included studies on binding to prostanoid receptors (DP_1, IP, EP_1, EP_2, EP_3 and EP_4 receptors) that are activated by TRE and involved with relaxation of pulmonary vascular smooth muscle (Walch et al., 1999; Foudi et al., 2008; Lai et al., 2008; Orie and Clapp, 2011; Whittle et al., 2012). We found that TRE bound to DP_1, IP, EP_1, EP_2 and EP_4 receptors with a rank order potency similar to that previously described (Whittle et al., 2012). In these assays, C16TR (10 μM) had no activity and also was inactive in a general battery of receptor binding and enzyme inhibition screens (data not shown). C16TR was weakly active in a

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platelet aggregation assay that previously demonstrated potent effects with TRE (Moncada et al., 1978; Whittle et al., 1978). These results demonstrate that C16TR possess no inherent pharmacological activity and is consistent with structure-activity data on the human prostacyclin receptor that identify the carboxy group at the C1 position as critical for ligand binding to the receptor (Stitham et al., 2003). In the synthesis of C16TR, the C1 position at the carboxy group is replaced with an ester bond that links the hexadecyl carbon chain to TRE (Leifer et al., 2014; Leifer et al., 2017). When the prodrug C16TR is cleaved by lung esterases (Leifer et al., 2014; Leifer et al., 2017), it also produces hexadecanol which is expected to be readily metabolized by pulmonary pneumocytes (Frenkel et al., 1993); note that hexadecanol was a constituent of the artificial pulmonary surfactant Exosurf® (Durand et al., 1985).

In vivo evaluations in hypoxia-exposed pulmonary hypertensive rats have identified several important features that distinguish inhaled C16TR-LNP from TRE. First, there was an immediate reduction in PAP after inhalation of TRE whereas the reduction in PAP with inhaled C16TR took several minutes to develop. Second, the reductions in PAP persisted through 180 min following inhalation of C16TR-LNP but not with inhaled TRE. We have recently confirmed and extended our observations on the prolonged pulmonary vasodilator activity of C16TR-LNP in rats and dogs in which C16TR-LNP was delivered by nose only inhalation followed by challenge with U46619 and hypoxia to induce pulmonary vasoconstriction (Li et al., 2016; Malinin et al., 2017). The results demonstrate an inhibition of pulmonary vasoconstriction up to 24 h after a single inhaled dose of C16TR-LNP. The actions of C16TR-LNP producing pulmonary vasodilation are likely due to the slow sustained release of TRE from C16TR-LNP following cleavage of the ester bond by the actions of endogenous lung esterase(s). This conclusion is supported by data with C16OTR, an ether-linked C16TR compound that is unaffected by the enzymatic actions of endogenous esterase (Blaner et al., 1984) and showed no activity to inhibit hypoxia-induced pulmonary vasoconstriction in rats.

There were also important differences in the PK profile between inhaled C16TR-LNP and TRE. In the efficacy studies measuring reductions in PAP in hypoxia-challenged rats, plasma TRE C_{max} was approximately 16-fold higher after inhaled TRE compared to

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C16TR-LNP. In these studies, plasma TRE decreased over 3 h after inhaled TRE but slowly increased after C16TR-LNP. In dogs, the plasma TRE C_{max} was 14-fold lower after C16TR-LNP (18 μg/kg) and sustained over 24 h whereas plasma TRE quickly disappeared after the same inhaled dose of TRE. In single dose and 14-day repeat dose studies with C16TR-LNP in rats, dose-dependent increases in plasma TRE C_{max} and AUC were found with no evidence of drug accumulation in the plasma or lungs with repeat dosing. TRE in the plasma and lungs was slowly eliminated after inhaled C16TR-LNP in rats with a first order exponential decline over 24 h. Similar results were found with shorter chain TRE prodrugs in rats (C12TR-LNP, C14TR-LNP) (Malinin et al., 2014). The slow elimination of TRE from the lungs has important implications for efficacy studies because a "localized" pulmonary vasodilator action of TRE in the lungs has been previously reported for continuously inhaled TRE in sheep (Sandifer et al., 2005) and more recently with inhaled C16TR-LNP in rats and dogs (Li et al., 2016; Malinin et al., 2017). A localized effect occurs when pulmonary vasodilation is observed but plasma levels are below the levels necessary for injected (systemic) TRE to achieve pulmonary vasodilation. Because many of the adverse events correlate with plasma levels, the implications of reduced systemic exposure may be significant.

Cough is frequently seen with inhaled TRE in human subjects and limits the dose and the frequency of administration of this drug (Nadler and Edelman, 2010; Channick, et al., 2012). Pulmonary C fibers are likely to be involved as they are activated by prostacyclin (Mapp et al., 1991; Ishiura et al., 2007) and are the same receptors involved with the cough response to another prostaglandin, PGE_2, in guinea pigs (Maher et al., 2009; Maher and Belvisi, 2010). To test the hypothesis that reduced cough would be observed with C16TR-LNP, experiments were performed in guinea pigs which is a species that duplicates many of the features of cough in humans (Morice et al., 2007; Canning, 2008; Maher et al., 2009). At a nebulized concentration of 30 μg/mL, inhaled TRE produced a consistent cough response whereas inhaled C16TR-LNP did not. Similar findings were recently found with C16TR-LNP delivered in a dry powder formulation that did not cause cough whereas nebulized TRE at 10 and 30, but not at 3

µg/mL produced a consistent cough response (Chapman et al., 2017). It is important to note that TRE-related side effects in dogs such as cough and rapid shallow breathing were only seen at the highest inhaled dose (95 µg/kg) of C16TR.

A phase 1 study has been conducted in healthy volunteers to determine the safety, tolerability and pharmacokinetics of escalating doses of C16TR-LNP relative to Tyvaso® (Han et al., 2016 a, b). The results demonstrate that at the lowest dose of 85 µg, inhaled C16TR-LNP had a lower plasma TRE C_{max}, sustained levels of TRE in the plasma over 12 h with no incidence of cough and throat irritation compared to inhaled Tyvaso® (54 µg). Higher doses of C16TR-LNP (170 and 340 µg) were well tolerated and measurable levels of TRE were detected in the plasma up to 24 h after single dose administration. Similar results were obtained in our preclinical studies with C16TR-LNP which demonstrate sustained levels of TRE in the plasma over 12-24 h in rats and dogs and no indication of cough in guinea pigs. These results demonstrate there is good translatability between the preclinical and clinical paradigms and based upon other preclinical studies showing a prolonged “localized” pulmonary vasodilator effect in the lungs (Li et al., 2016; Malinin et al., 2017) we believe that C16TR-LNP has the potential for once-daily, convenient dosing in humans.

In summary, these preclinical studies identify inhaled C16TR-LNP as a long-acting pulmonary vasodilator that involves the slow, sustained release of TRE from C16TR-LNP. C16TR is devoid of inherent pharmacological activity and had no direct effect to inhibit platelet aggregation. The PK properties of C16TR-LNP in rats and dogs with low plasma TRE C_{max} and sustained levels of TRE in the lungs support the concept of a “localized” effect in the lungs contributing to its prolonged pulmonary vasodilator activity. Inhaled C16TR-LNP was well tolerated in rats, dogs and guinea pigs and there was no evidence of TRE accumulation in the blood and lungs after repeat dosing. Inhaled C16TR did not cause cough in guinea pigs. In conclusion, the attributes of inhaled C16TR-LNP described herein offer an advantage over inhaled TRE with regard to a longer interval between dosing and a reduced potential for side effects.

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AUTHOR CONTRIBUTIONS

Participated in research design: Corboz, Li, Malinin, Perkins, Chapman

Conducted experiments (platelet aggregation, hypoxia challenge in rats, PK in rats): Yin, Laurent, Salvail

Conducted experiments (cough reflex in guinea pigs, PK in dogs): Xu, Zhuang, Curran

Performed data analysis: Li, Yin, Biernat, Laurent, Xu, Zhuang, Chen, Curran

Contributed new reagents and analytical tools: Konicek, Leifer, Plaunt

Wrote or contributed to the writing of the manuscript: Chapman, Perkins, Corboz

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: Chemical structures of C16TR and C16OTR.

Figure 2: Effect of C16TR formulated in squalane/DSPE-PEG2000 LNP and TRE on platelet aggregation induced by the addition of ADP (10 μM) to rat blood. Results are expressed as the % inhibition of platelet aggregation due to drug relative to values obtained in PBS controls. Values represent the mean ± standard error of the mean (SEM) from 2-3 determinations.

Figure 3: Dose-response pulmonary vasodilation with inhaled C16TR formulated in squalane/DSPE-PEG2000 LNP in hypoxia-challenged rats. Targeted pulmonary doses are shown for each group. Values represent the mean ± SEM % decrease from the hypoxic baseline (n = 3-4 per dose of C16TR-LNP, n = 10 for PBS). * $P<0.05$ compared C16TR-LNP to PBS.

Figure 4: Pulmonary vasodilator response to inhaled TRE and inhaled C16TR and C16OTR formulated in squalane/DSPE-PEG2000 LNP in hypoxia-challenged rats. Values represent the mean ± SEM % decrease from the hypoxic baseline (n = 3 for C16TR-LNP, n = 6 for C16OTR-LNP and n = 10 for TRE). * $P<0.05$ compared C16TR-LNP to TRE.

Figure 5: Concentrations of A) TRE in the plasma and B) combined TRE + C16TR (TRE equivalent concentration (TRE Eq)) in the lungs following nose-only inhalation of C16TR formulated in squalane/DOPC/cholesterol-PEG2000 LNP in rats. Data shows individual values for 3 rats at each of the 7 time points.

Figure 6: Combined TRE + C16TR (TRE Eq) in the lungs following nose-only inhalation of C16TR formulated in squalane/DSPE-PEG2000 LNP for 1, 7 or 14 days in rats. Lung PK measurements were made 24 h after the last dose. Values are the mean ± SEM (n = 4 per dose).

Figure 7: Concentration of TRE in the plasma of dogs after inhaled TRE and the TRE prodrugs formulated in squalane/DOPC/cholesterol-PEG2000 LNP. The dogs were exposed to TRE (16 µg/kg, n = 5), C12TR-LNP (19 µg/kg, n = 5), C14TR-LNP (21 µg/kg, n = 2) and C16TR-LNP (22 µg/kg, n = 5). Values are the mean ± SEM.

Figure 8: Concentration of TRE in the plasma of dogs after inhaled A) C16TR formulated in squalane/DOPC/cholesterol-PEG2000 LNP and B) TRE. The dogs were exposed to TRE (5 µg/kg, n = 3 and 16 µg/kg, n = 5) or C16TR-LNP (7 µg/kg, n = 3; 22 µg/kg, n = 5; 46 µg/kg, n = 3; 95 µg/kg, n = 3) and blood samples were collected over 72 h. Values are the mean ± SEM.

Figure 9: Effect of inhaled TRE and C16TR formulated in squalane/DSPE-PEG2000 LNP on cough in guinea pigs. The results are A) the total number of coughs and B) the number of cough bouts induced by inhaled TRE (30 µg/mL) and inhaled C16TR-LNP (30 µg/mL TREEq). Values are the mean ± SEM for TRE (n=7) and C16TR-LNP (n=6). * $p < 0.05$ compared to TRE.

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Table 1: Binding Activity of TRE and C16TR to Prostanoid Receptors.

	Inhibitory Concentration (IC_{50}, μM)	
Prostanoid Receptor	TRE	C16TR
EP_2	0.014	>10
DP_1	0.023	>10
IP	0.079	>10
EP_1	0.20	NT
EP_3	0.25	NT
EP_4	0.92	>10
$CRTH_2$	>10	NT
FP	>10	NT

Values are the mean from duplicate experiments.

NT-Not tested.

Table 2: Plasma Concentrations of TRE after Inhaled TRE and Inhaled C16TR Formulated in squalane/DSPE-PEG2000 LNP in Hypoxia-Challenged Rats.

Time After Start of Inhalation (min)	TRE (6 µg/kg) (n=10)	C16TR-LNP (6 µg/kg) (n=3)
3	3.44	0.06
20	2.07	0.07
40	0.90	0.13
60	0.50	0.14
90	0.31	0.18
180	0.44	0.22

Values are the mean plasma concentration of TRE (ng/mL).

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Table 3: TRE Plasma PK in Rats for Inhaled C16TR Formulated in squalane/DOPC/cholesterol-PEG2000 LNP.

Parameter	C16TR-LNP (µg/kg)			
	0.6	**1.8**	**6**	**18**
TRE Plasma C_{max} (ng/ml)	0.40	1.67	3.46	10.21
TRE Plasma T_{max} (h)	1	1	1	1
TRE Plasma $AUC_{0\text{-}24h}$ (ng*h/ml)	1.89	7.35	19.61	70.38

Values are the mean (n = 21/dose; 3 rats per time point; 7 time points over 24 h).

C_{max} mean maximum plasma concentration.

T_{max} time to maximum plasma concentration.

$AUC_{0\text{-}24h}$ area under the plasma TRE concentration curve over a 24 h period.

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Table 4: Plasma PK Parameters for TRE in Rats Following Once-Daily Administration of Inhaled C16TR Formulated in squalane/DSPE-PEG2000 LNP for up to 14 Consecutive Days.

C16TR PD (µg/kg)	Group	IBD ng/mL	C_{max} ng/mL	Average C_{max} ng/mL	AUC_{0-24} h*ng/mL	Average AUC_{0-24h} h*ng/mL	AUC/PD	Average AUC/PD
0.6	Day 1	NA	0.14	0.22	0.44	1.10	0.73	1.83
	Day 7	0	0.23		1.03		1.72	
	Day 14	0.015	0.28		1.83		3.05	
1.8	Day 1	NA	0.75	0.73	4.88	5.86	2.71	3.26
	Day 7	0.042	0.82		7.89		4.38	
	Day 14	0.069	0.63		4.82		2.68	
6	Day 1	NA	0.72	1.66	4.15	11.15	0.69	1.86
	Day 7	0.042	2.79		18.5		3.09	
	Day 14	0.093	1.48		10.8		1.80	
18	Day 1	NA	6.48	5.46	50.4	48.33	2.80	2.69
	Day 7	0.297	5.18		48.0		2.67	
	Day 14	0.190	4.71		46.6		2.59	

Values are the mean plasma concentration of TRE (ng/ml) (n = 4 per dose).

Abbreviations: PD, Pulmonary Dose; IBD, Immediately Before Dose; NA; Not Applicable; C_{max}, mean maximum plasma concentration; $AUC_{0-24h,}$ area under the plasma TRE concentration curve over a 24 h period.

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Table 5: Plasma PK of TRE and Clinical Signs in Dogs after Administration of Inhaled TRE or Inhaled C12TR, C14TR and C16TR Formulated in squalane/DOPC/cholesterol-PEG2000 LNP.

Compound	n	Targeted Pulmonary Dose (µg/kg)	Delivered Pulmonary dose (µg/kg)	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-24h} (ng*h/mL)	AUC_{0-72h} (ng*h/mL)
TRE	3	6	5	2.7	0.1	1.71	1.71
	5	18	16*	5.5	0.2	5.08	5.08
C12TR-LNP	5	18	19*	1.2	1	4.20	4.98
C14TR-LNP	2	18	21	0.4	1	2.91	4.44
	3	95	93*	1.5	1	12.45	16.10
C16TR-LNP	3	6	7	0.2	0.1	0.97	1.35
	5	18	22	0.4	0.02	2.08	3.34
	3	40	46	0.9	0.02	4.22	6.71
	3	95	95*	1.5	0.1	11.67	18.98

Values represent the mean values.

Abbreviations: C_{max}, mean maximum plasma concentration; T_{max}, Time to maximum plasma concentration; $AUC_{0-24h,}$ area under the plasma TRE concentration curve over a 24 h period; $AUC_{0-72h,}$ area under the plasma TRE concentration curve over a 72 h period.

* Clinical signs of cough, rapid shallow breathing, emesis and pale gums.

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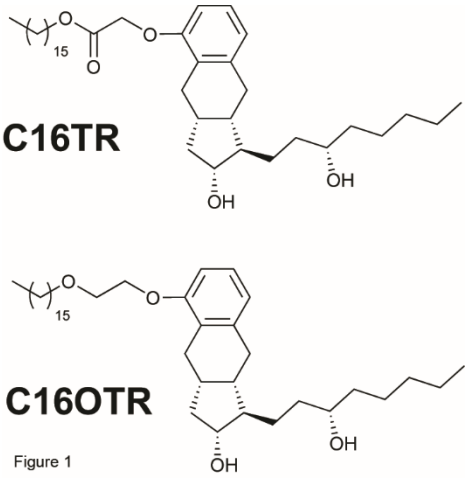

Figure 1

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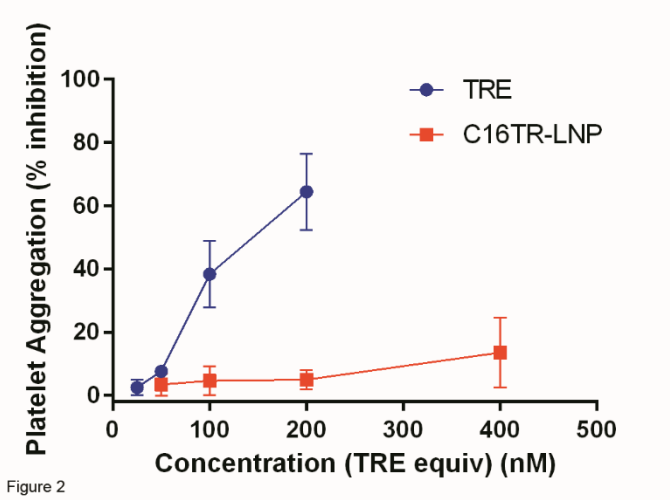

Figure 2

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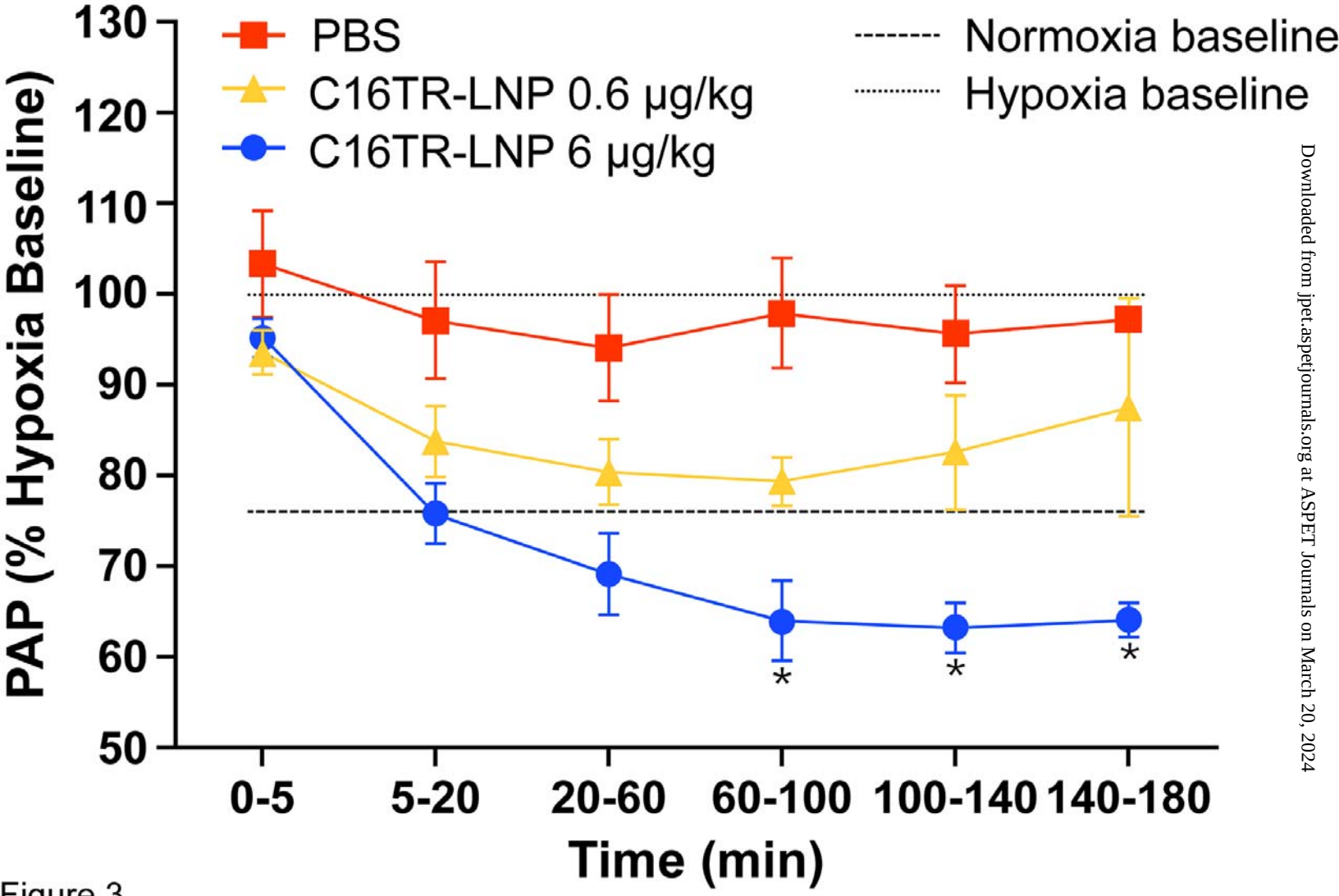

Figure 3

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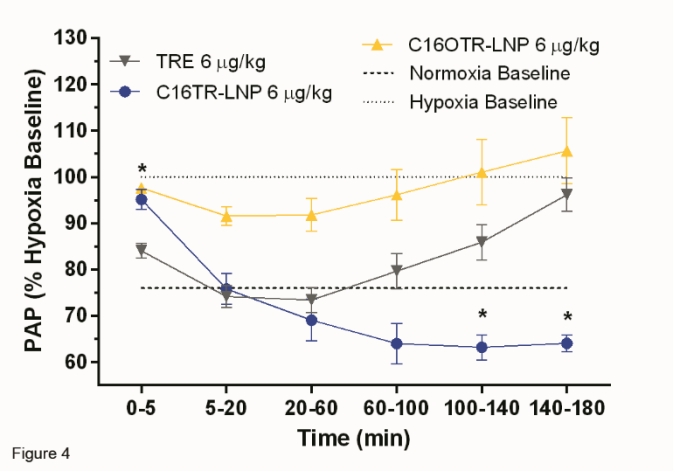

Figure 4

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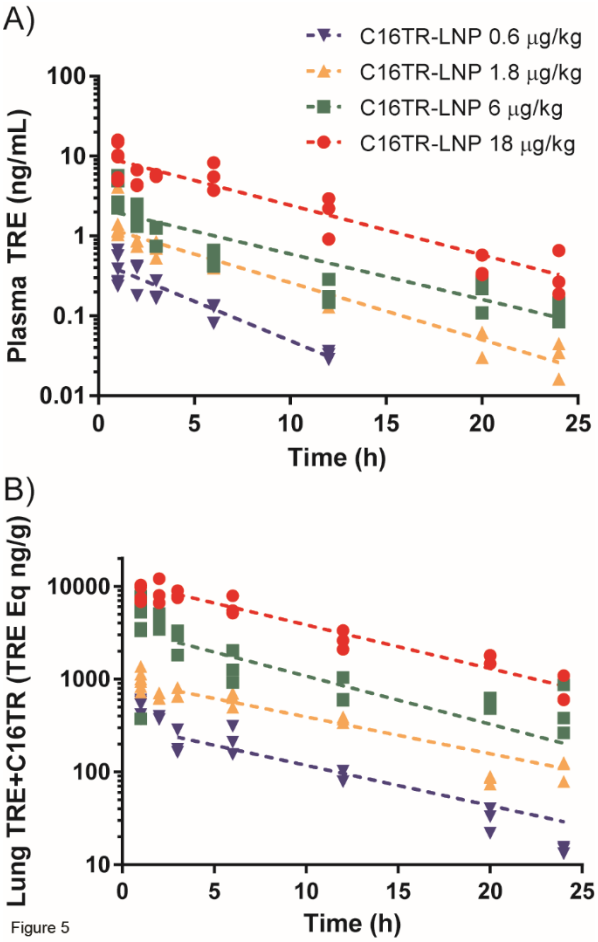

Figure 5

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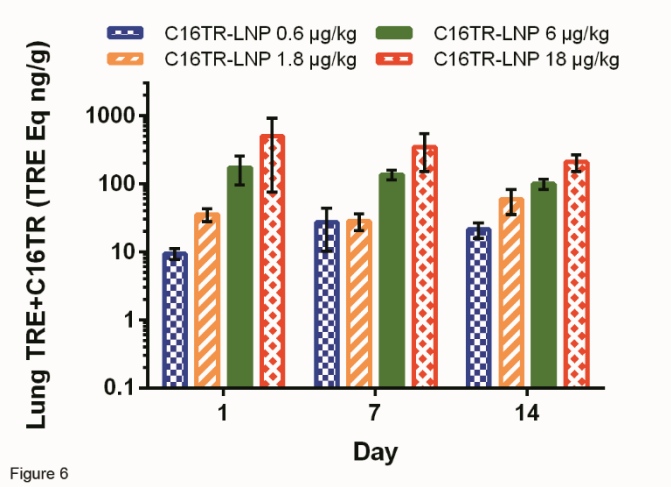

Figure 6

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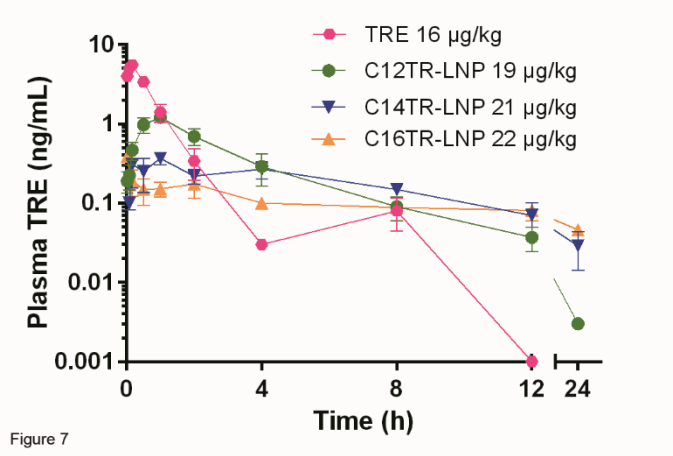

Figure 7

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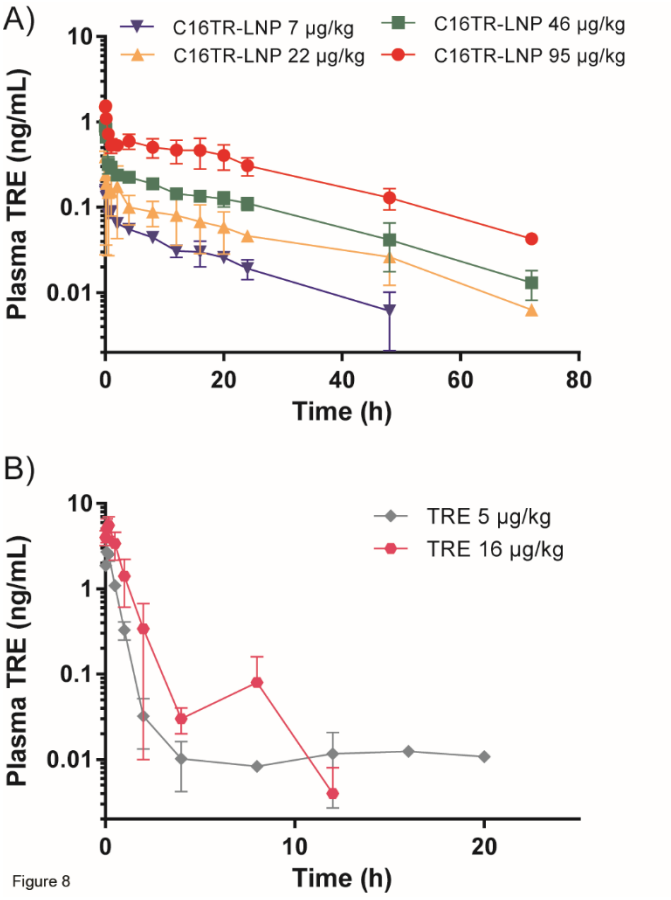

Figure 8

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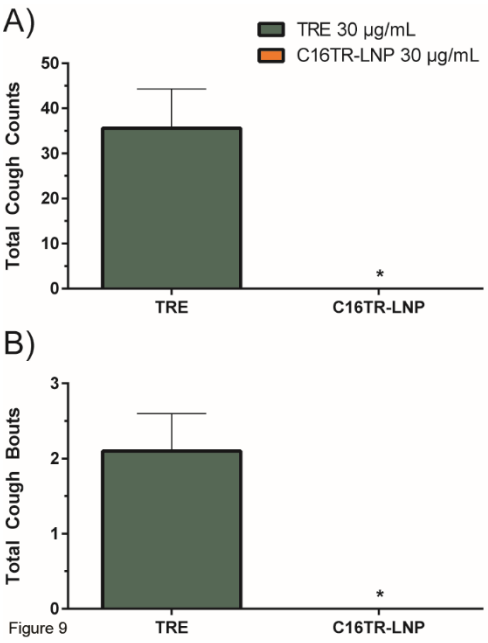

Figure 9