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


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ABSTRACT

This article 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 lower activity (>10 μM) in receptor binding and enzyme inhibition assays, including binding to prostaglandin E2 receptor 2, prostaglandin D2 receptor 1, prostaglandin I2 receptor, and prostaglandin E2 receptor 4; TRE potently bound to each of these prostanoid receptors. C16TR had no effect (up to 200 nM) on platelet aggregation induced by ADP in rat blood. In hypoxia-challenged rats, inhale C16TR-LNP produced dose-dependent (0.06–6 μg/kg) sustained pulmonary vasodilation over 3 hours; inhaled TRE (6 μg/kg) was active at earlier times but lost its effect by 3 hours. Single- and multiple-dose PK studies of inhaled C16TR-LNP in rats showed proportionate dose-dependent increases in TRE Cmax and area under the curve (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 Cmax compared with inhaled TRE. Inhaled C16TR-LNP was well tolerated in rats and dogs; TRE-related side effects included cough, respiratory tract irritation, and emesis and were seen only 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, progressive disease that is characterized by the constriction and remodeling of the pulmonary vasculature, leading to increased pulmonary vascular resistance and pulmonary arterial pressure (PAP), most often resulting in right-sided heart failure (Stamm et al., 2011; Frumkin, 2012). Current therapies to treat PAH have been directed toward 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).

ABBREVIATIONS: AUC, area under the plasma curve; C12TR, dodecyl-treprostinil; C14TR, tetradecyl-treprostinil; C16OTR, ether-linked hexadecyl-treprostinil derivative; C16TR, hexadecyl-treprostinil; Cmax, mean maximum plasma concentration; CRTH2, chemoattractant receptor-homologous molecule expressed on T-helper cells; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DP1, prostaglandin D2 receptor 1; DSPE-PEG2000, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000; EP1, prostaglandin E2 receptor 1; EP2, prostaglandin E2 receptor 2; EP4, prostaglandin E2 receptor 4; F2α, prostaglandin F2α receptor; HPLC/UV, high-performance liquid chromatography/ultraviolet; HR, heart rate; INS1009, hexadecyl-treprostinil formulated in a lipid nanoparticle; IP, prostaglandin I2 receptor; K2-EDTA, Di-potassium ethylenediaminetetraacetic acid; LLOQ, lower limit of quantitation; LNP, lipid nanoparticle; PAH, pulmonary arterial hypertension; PAP, pulmonary arterial pressure; PBS, phosphate-buffered saline; PK, pharmacokinetics; SaO2, arterial oxygen saturation; SAP, systemic arterial blood pressure; T1/2, time to maximum plasma concentration; TRE, treprostinil.
Treprostinil (TRE) is a relatively stable analog 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. Remodulin administration is associated with significant injection site pain (s.c.) or concern of infection (i.v. administration) (Simonneau et al., 2002; Remodulin, 2014); however, Orenitram and Tyvaso require multiple doses daily, and efficacy may not be fully maintained over a 24-hour period (Channick et al., 2012; Tyvaso, 2013; Orenitram, 2014; Feldman et al., 2015). Inhaled TRE is also associated with numerous 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 four times daily (Voswinckel et al., 2006; Nadler and Edelman, 2010; Channick et al., 2012; Tyvaso, 2013).

Sandifer et al. (2005) demonstrated that continuously inhaled TRE showed greater efficacy in sheep with fewer systemic side effects than continuously infused drug; however, in that study, inhaled TRE had to be administered continuously 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 vasodilatation 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, unpublished). The culmination of this research identified hexadecyl-treprostinil (C16TR) as a lead inhalation prodrug candidate.

Here 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 (Leifer et al., 2014; Malinin et al., 2014, 2015, 2017; Chapman et al., 2015, 2017; Li et al., 2016). The experiments performed and described herein are the following: 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.

### Materials and Methods

**Materials.** We obtained TRE from Chirogate International (Taoyuan County, Taiwan, Republic of China) and dodecanol, tetradecanol, hexadecanol, 1,4 dioxane, amylloxybenzoylamine-N-methoxy(polyethylene glycol)-2000] (DSPE-PEG2000, ammonium salt) and cholesterol-PEG2000 were obtained from NOF America (White Plains, NY). Dodecyl-TRE (C12TR), tetradecyl-TRE (C14TR), and C16TR were synthesized at Insmed Incorporated (Bridgewater, NJ) 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, unpublished). Phosphate-buffered saline (PBS) was acquired from Mediatech (Manassas, VA). 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; Malinin et al., 2015, 2017; Han et al., 2016 a,b; Li et al., 2016). The other formulation contains C16TR and squalane, but with 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) and cholesterol-PEG2000; the molar ratio of these four ingredients was 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, which 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 C16TR/squalane/DSPE-PEG2000 in a molar ratio of 45:45:10. The chemical structures of C16TR and C16OTR are shown in Fig. 1.

**Radioligand Binding Assays.** Radioligand binding assays for human prostaglandin D₂ receptor 1 (Dp₁), prostaglandin I₂ receptor (IP), prostaglandin E₂ receptor 1 (EP₁), prostaglandin E₂ receptor 2 (EP₂), prostaglandin E₂ receptor 3 (EP₃), prostaglandin E₂ receptor 4 (EP₄), prostaglandin F₂α receptor (FP), and chemoattractant receptor homologous molecule expressed on T-helper cells (CRTH₂) receptors were performed by Eurofins Panlabs (Taipei, Taiwan, Republic of China). The following radioligands were used: [³H] prostaglandin D₂, [³H] CRTH₂, [³H] prostaglandin E₂, [³H] EP₂, [³H] EP₃, [³H] EP₄, [³H] FP, and [³H] iloprost (IP). The compounds were incubated at 25°C for either 1 hour (EP₁, FP, and IP) or 2 hours (DP₁, CRTH₂, EP₂, EP₃, and EP₄) with human recombinant Chinese hamster ovary K1 cell line cells expressing the appropriate human prostanoid receptor. Binding activity was measured as the inhibition (%) of radioligand binding, and IC₅₀ values were determined by a nonlinear 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 the availability of the prodrug in this assay.

**Platelet Aggregation.** Blood samples from male Sprague Dawley rats (Charles River Laboratories, QC, Canada) were centrifuged to extract platelet-rich plasma and platelet-poor plasma. Platelet-rich plasma was diluted with platelet-poor plasma to yield samples

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**Fig. 1.** Chemical structures of C16TR and C16OTR.
containing 3.5 × 10⁶ platelets/ml. Platelet aggregation was induced by the addition of 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 percentage of inhibition attributable to drug or control.

Hypoxia Challenge in Rats. Male Sprague Dawley rats (Charles River Laboratories, Quebec, Canada) ranging in weight from 300 to 350 g were anesthetized with 3% isoflurane-oxygen and then transitioned to 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; 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 saturation (SaO2). 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.

After measurement of PAP, SAP, HR, and SaO2 during ventilation on room air (21% O2), the inspired gas was switched to a hypoxic gas mixture (10% O2) and maintained at hypoxic levels (SaO2 ≈ 45–60%) for the duration of the study. Once a 5-minute stable elevation in PAP had been achieved during the hypoxia exposure, physiologic parameters, including SAP, 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 physiologic parameters were measured over a 180-minute period. The data were sampled over 20-second periods (200 data points per second) and averaged as a single value at times of 0–5, 5–20, 20–60, 60–100, 100.3–147, and 147.7–180 minutes after nebulization of the drugs. The changes induced by the test articles were measured at each of these time intervals and expressed as a percentage of 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 minutes after drugs, and lungs were harvested at the end of the studies (i.e., 180 minutes). The concentration of TRE and C16TR was measured by collecting drug deposited on a filter attached to the end of the endotracheal tube per unit of time. Inhaled drug dose (micrograms per kilograms) was measured from the relationships of aerosol drug concentration, the duration of drug exposure, and minute ventilation based on 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 hours 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 (K₂EDTA) anticoagulant and put immediately on ice until processed. Processing occurred within 2 hours of blood collection. The samples were centrifuged (for 10 minutes at approximately 2000g at 2 to 8°C) to obtain plasma. Plasma (approximately 500 μl for each aliquot) was stored frozen at nominal −10°C, and the concentration of TRE was measured using a qualified HPLC/MS/MS method. The plasma TRE samples were used to determine Cmax, Tmax, and AUC0–24 h for each dosing interval.

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 an 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 or C14TR was used as internal standard for C16TR. Extracts were analyzed using Ace 3 C18 (Kinetex phenylhexyl) 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).

**Cough Reflex in Guinea Pigs.** Cough was measured in conscious, unrestrained guinea pigs (250–350 g) (Charles River Laboratories Inc., Wilmington, MA) 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 (n = 1), 3.3 (n = 3), 10 (n = 4), 30 (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 minutes. 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 minutes, and the total number of coughs was measured during the 10 minutes of nebulization and for an additional 20-minute period after the test article was given. Airflow through the plethysmograph was maintained at a constant flow rate of 2 liters/min and was derived from a compressed air source. Statistical significance between the treatment groups was determined using Kruskal-Wallis analysis of variance (nonparametric) followed by the Dunn method for joint ranking (Kruskal and Wallis, 1952; Dunn, 1961).

**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, QC, Canada; Lovelace Respiratory Research Institute, Albuquerque, New Mexico; and Envigo CRS Inc., East Millstone, NJ. These institutions are facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

**Results**

**Binding to Prostanoid Receptors.** TRE had binding to the following prostanoid receptors with an order of potency (estimated IC50) of EP2 (0.014 μM) > DP1 (0.023 μM) > IP (0.079 μM) > EP1 (0.20 μM) > EP3 (0.25 μM) > EP4 (0.92 μM); TRE had no activity (>10 μM) against CRTH2 and FP (Table 1). In contrast, C16TR had no binding (>10 μM) to these prostanoid receptors. Similar results were found with C16OTR, which had no binding (>10 μM) to these prostanoid receptors.

TABLE 1

<table>
<thead>
<tr>
<th>Prostanoid Receptor</th>
<th>Inhibitory Concentration (IC50, μM)</th>
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<tr>
<td></td>
<td>TRE</td>
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<tr>
<td>EP2</td>
<td>0.014</td>
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<tr>
<td>DP1</td>
<td>0.023</td>
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<tr>
<td>IP</td>
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<td>EP1</td>
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<td>EP3</td>
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<td>EP4</td>
<td>0.92</td>
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<tr>
<td>CRTH2</td>
<td>NT</td>
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<tr>
<td>FP</td>
<td>&gt;10</td>
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NT, Not tested.

**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 (Fig. 2). The LNP vehicle minus C16TR had no effect (data not shown).

**Hypoxia Challenge in Rats.** After challenge with hypoxia, PAP increased from values of 17.8 ± 0.6 mm Hg during normoxia to 23.2 ± 0.8 mm Hg during hypoxia (n = 32). This elevation in PAP did not appreciably change during the 180 minutes of hypoxia exposure and was unaffected by exposure to nebulized PBS (Fig. 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

![Fig. 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 percent inhibition of platelet aggregation from drug relative to values obtained in PBS controls. Values represent the mean ± S.E.M. from two to three determinations.](image1)

![Fig. 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 ± S.E.M. percent decrease from the hypoxic baseline (n = 3 to 4 per dose of C16TR-LNP, n = 10 for PBS). *P < 0.05 compared C16TR-LNP with PBS.](image2)
reduced to 13 ± 1 mm Hg, which 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 (Fig. 4). A higher dose of TRE (10 μg/kg, n = 4) produced no further reduction in PAP (normoxia, PAP = 18.6 ± 1.6 mm Hg; hypoxia, PAP = 25.1 ± 2.6 mm Hg; hypoxia + TRE, PAP = 18.0 ± 1.8 mm Hg). No changes in SAP and HR were noted with any treatments (data not shown).

Two important differences were seen in the pulmonary vasodilator responses of inhaled C16TR-LNP and inhaled TRE (Fig. 4). First, a reduction in PAP occurred during nebulization with inhaled TRE (6 μg/kg) and was already 15% below the baseline values by 5 minutes after challenge, whereas the reduction in PAP for inhaled C16TR-LNP (6 μg/kg) was delayed and not observed until 5–20 minutes after administration (Fig. 4). Second, over 180 minutes, 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) (Fig. 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 minutes in this model of hypoxia-challenged rats.

Plasma concentrations of TRE were highest (3.44 ng/ml) by 3 minutes 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 minutes and slowly increased to the highest value of 0.22 ng/ml at 180 minutes (Table 2). In the lung, the TRE-equivalent concentration (C16TR = TRE; see Materials and Methods) was 67-fold higher for inhaled C16TR-LNP (438 ng/g lung tissue) than for 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 (Fig. 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_{0–24h} for inhaled C16TR-LNP with a plasma TRE T_{max} at 1 hour at all doses (Table 3). The concentration of TRE and C16TR also increased in the lungs with increasing inhaled doses of C16TR-LNP (Fig. 5B), with most of the combined TRE + C16TR (TRE equivalent...
Inhaled C16TR-LNP produced dose-dependent increases in plasma TRE with levels of TRE detected up to 72 hours after inhalation for doses of 46 and 95 μg/kg (Fig. 8A). Corresponding values for plasma TRE $C_{\text{max}}$ and AUC $0\rightarrow24\ h$ 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 by 24 hours (Fig. 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 cough in all guinea pigs (seven of seven) 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 as a result of adverse effects. PBS was also administered to six 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 ± coughs; range, 17–82 coughs); coughing occurred with one to four 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 (Fig. 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 because of its relatively short duration of action and the number of adverse side effects it evokes (Voswinckel et al., 2006; Nadler and Edelman, 2010; Channick et al., 2012).

To improve the duration of action and reduce the side effects of TRE, LNP formulations of C16TR were developed, resulting in...
a lead compound, INS1009 (Leifer et al., 2017, unpublished). In this report, pharmacologic and PK studies were performed to highlight some of the important features of inhaled C16TR-LNP. We found that C16TR possessed no inherent pharmacologic 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 owing to the slow conversion of TRE from C16TR present in the lungs. Lower plasma TRE \( C_{\text{max}} \) was found with inhaled C16TR-LNP compared with inhaled TRE in rats and dogs, and no evidence of drug accumulation was seen 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 with inhaled TRE.

To establish whether C16TR can be classified as an inactive prodrug, evaluations were performed in receptor binding and enzyme inhibition assays, including 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 those previously described (Whittle et al., 2012). In these assays, C16TR (10 \( \mu M \)) 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 platelet aggregation assay that previously demonstrated potent effects with TRE (Moncada et al., 1978; Whittle et al., 1978). These results demonstrate that C16TR possesses no inherent pharmacologic activity and are 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; 2017, unpublished).

When the prodrug C16TR is cleaved by lung esterases (Leifer et al., 2014, 2017, unpublished), 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 (collosceril palmitate) (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 minutes after 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 hours after a single inhaled dose of C16TR-LNP. The actions of C16TR-LNP producing pulmonary vasodilation are likely owing to the slow sustained release of TRE from C16TR-LNP after 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 of inhaled C16TR-LNP and TRE. In the efficacy studies measuring reductions in PAP in hypoxia-challenged rats, plasma TRE \( C_{\text{max}} \) was approximately 16-fold higher after inhaled TRE compared with C16TR-LNP. In these studies, plasma TRE decreased over 3 hours after inhaled TRE but slowly increased after C16TR-LNP. In dogs, the plasma TRE \( C_{\text{max}} \) was 14-fold lower after C16TR-LNP (18 \( \mu g/kg \)) and sustained over 24 hours, 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 $C_{\text{max}}$ 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 hours. 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 (i.e., prostaglandin E$_2$) 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, 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 \( \mu g/ml \), 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 \( \mu g/ml \), 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 seen only at the highest inhaled dose (95 \( \mu g/kg \)) of C16TR.

A phase 1 study has been conducted in healthy volunteers to determine the safety, tolerability, and PK 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 \( \mu g \), inhaled C16TR-LNP had a lower plasma TRE $C_{\text{max}}$, sustained levels of TRE in the plasma over 12 hours with no incidence of cough, and throat irritation compared with inhaled Tyvaso (54 \( \mu g \)). Higher doses of C16TR-LNP (170 and 340 \( \mu g \)) were well tolerated with no reported adverse events.

### Table 5

<table>
<thead>
<tr>
<th>Compound</th>
<th>$n$</th>
<th>Targeted Pulmonary Dose ($\mu g/kg$)</th>
<th>Delivered Pulmonary Dose ($\mu g/kg$)</th>
<th>$C_{\text{max}}$ (ng/mL)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>AUC$_{0-24\text{h}}$ (ng*h/mL)</th>
<th>AUC$_{0-72\text{h}}$ (ng*h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRE</td>
<td>3</td>
<td>6</td>
<td>5</td>
<td>2.7</td>
<td>0.1</td>
<td>1.71</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>18</td>
<td>16(^a)</td>
<td>5.5</td>
<td>0.2</td>
<td>5.08</td>
<td>5.08</td>
</tr>
<tr>
<td>C12TR-LNP</td>
<td>5</td>
<td>18</td>
<td>19(^a)</td>
<td>1.2</td>
<td>1</td>
<td>4.20</td>
<td>4.98</td>
</tr>
<tr>
<td>C14TR-LNP</td>
<td>2</td>
<td>18</td>
<td>21</td>
<td>0.4</td>
<td>1</td>
<td>2.91</td>
<td>4.44</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>95</td>
<td>93(^a)</td>
<td>1.5</td>
<td>1</td>
<td>12.45</td>
<td>16.10</td>
</tr>
<tr>
<td>C16TR-LNP</td>
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<td>6</td>
<td>7</td>
<td>0.2</td>
<td>0.1</td>
<td>0.97</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>5</td>
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<td>22</td>
<td>0.4</td>
<td>0.02</td>
<td>2.08</td>
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<tr>
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<td>40</td>
<td>46</td>
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<td>0.02</td>
<td>4.22</td>
<td>6.71</td>
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<tr>
<td></td>
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<td>95</td>
<td>95(^a)</td>
<td>1.5</td>
<td>0.1</td>
<td>11.67</td>
<td>18.98</td>
</tr>
</tbody>
</table>

\(^a\)Clinical signs of cough, rapid shallow breathing, emesis, and pale gums.
tolerated, and measurable levels of TRE were detected in the plasma up to 24 hours 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 hours in rats and dogs and no indication of cough in guinea pigs. These results demonstrate good translatability between the preclinical and clinical paradigms and based on other preclinical studies showing a prolonged "localized" pulmonary vasodilator effect in the lungs (Li et al., 2016; Malinin et al., 2017), we believe 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 pharmacologic activity and had no direct effect to inhibit platelet aggregation. The PK properties of C16TR-LNP in rats and dogs with low plasma TRE $C_{\text{max}}$ 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.

**References**


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