Supplementary Information

Inhaled Treprostinil-Prodrug Lipid Nanoparticle Formulations Provide Long-Acting Pulmonary Vasodilation

Authors
Franziska G. Leifer[1], Donna M. Konicek[1], Kuan-Ju Chen[1], Adam J. Plaunt[1], Dany Salvail[2], Charles E. Laurent[2], Michel R. Corboz[1], Zhili Li[1], Richard W. Chapman[1], Walter R. Perkins[1], Vladimir Malinin[1*]

Affiliations
[1]Insmed Incorporated, Bridgewater, NJ, USA
[2]IPS Therapeutique Inc., Sherbrooke, QC, Canada

***Corresponding author:**
Vladimir Malinin, PhD
10 Finderne Ave
Building 10
Bridgewater, NJ 08807
Tel.: +1/908/947 4334
Fax: +1/908/526 4026
Email: Vladimir.malinin@insmed.com

SUPPLEMENTAL METHODS

Routine maintenance of cell-cultures

CHO-K1 (Chinese Hamster Ovary cells) (ATCC CCL-61) used for the studies were obtained from ATCC. CHO-K1 cells were routinely maintained at < 90% confluent in F12 media (Life Technologies, Cat #: 31765092) supplemented with 10% fetal bovine serum (FBS, Hyclone, Cat #: SH30071.03), 1X Penicillin-Streptomycin (Life Technologies, Cat #: 15140-122) and kept in a 37° C Water-Jacketed incubator with 5% CO_2.

Transfections

For transfection studies, CHO-K1 cells were harvested when the cell monolayer reached 50-90% confluent. Briefly, cells were trypsinized with 0.05% Trypsin-EDTA (Life Technologies, cat #: 2530054) and dislodged by gently tapping the flask on the side. Trypsinzed cells were then re-suspended in 10mL of antibiotic free growth media (Life Technologies, Cat #: 31765092) containing 10% fetal bovine serum (FBS; Hyclone, Cat #: SH30071.03) and centrifuged at 250 x g for 5 minutes at room temperature. Cell pellet were re-suspended in 10 mL of antibiotic free growth media and cell number were determined using hemocytometer. 2.35e6 cells were seeded in 23.5mL of antibiotic free growth media (Life Technologies, Cat #: 31765092) containing 10% fetal bovine serum (FBS; Hyclone, Cat #: SH30071.03) per 75cm2 flask (BD Falcon, cat # 353136) and incubated overnight in a 37° C Water-Jacketed incubator with 5% CO2. Cells are ready for transfections. 235ug of pGloSensorTM -22F: 117.5ng of EP2-

Receptor: 117.5ng of pGEM3Zf(+) were diluted in Opti-MEM serum medium (Life Technologies, Cat #: 1985062) such that the final concentrations of the DNA was 12.6ng/µl. Next, 70.5ul of Fugene HD transfection reagent (Promega, Cat #: E2311) were added to 1880ul of diluted DNA plasmids and mixed carefully by pipetting. The complex was incubated at room temperature for 0-10 minutes and then transferred into each 75cm^2 flask containing overnight cultures of cells. The flasks were gently swirled and incubated for 20-24 hours in a 37° C Water-Jacketed incubator with 5% CO2.

pGloSensorTM cAMP Assay Reagents and Procedures

Following an overnight incubation in a 37° C Water-Jacketed incubator with 5% CO2 (for freshly transfected or overnight thawed transfected cells), the plates were removed and cell media replaced with 100µl of equilibration media per well. The equilibration media was prepared by diluting the GloSensorTM cAMP Reagent (Promega, Cat #: E1291) to 6 % v/v in 84% CO2-independent media (Life Technologies, Cat #: 18045088) and 10% FBS (Hyclone, Cat #: SH30071.03). The assay plate was incubated at room temperature for 2 hours to allow a steady-state basal signal to be reached. Luminescence was read using the Microlumat Plus reader to determine the first pre-read measurement. For samples with IBMX (Sigma, Cat #: I7018) treatment, 10µl of IBMX at working solution of 10X in plain media were added per well resulting in final concentration of 100µM. Appropriate controls were added to samples without IBMX treatment. The plate was incubated for an additional 10 minutes and a second pre-read measurement was taken. Following that, 10µl of test compounds at working solution of 10X in plain media were added per well resulting in final concentration of 1X. The plate was read every 5 minutes for the duration of the assay.

MMAD measurements

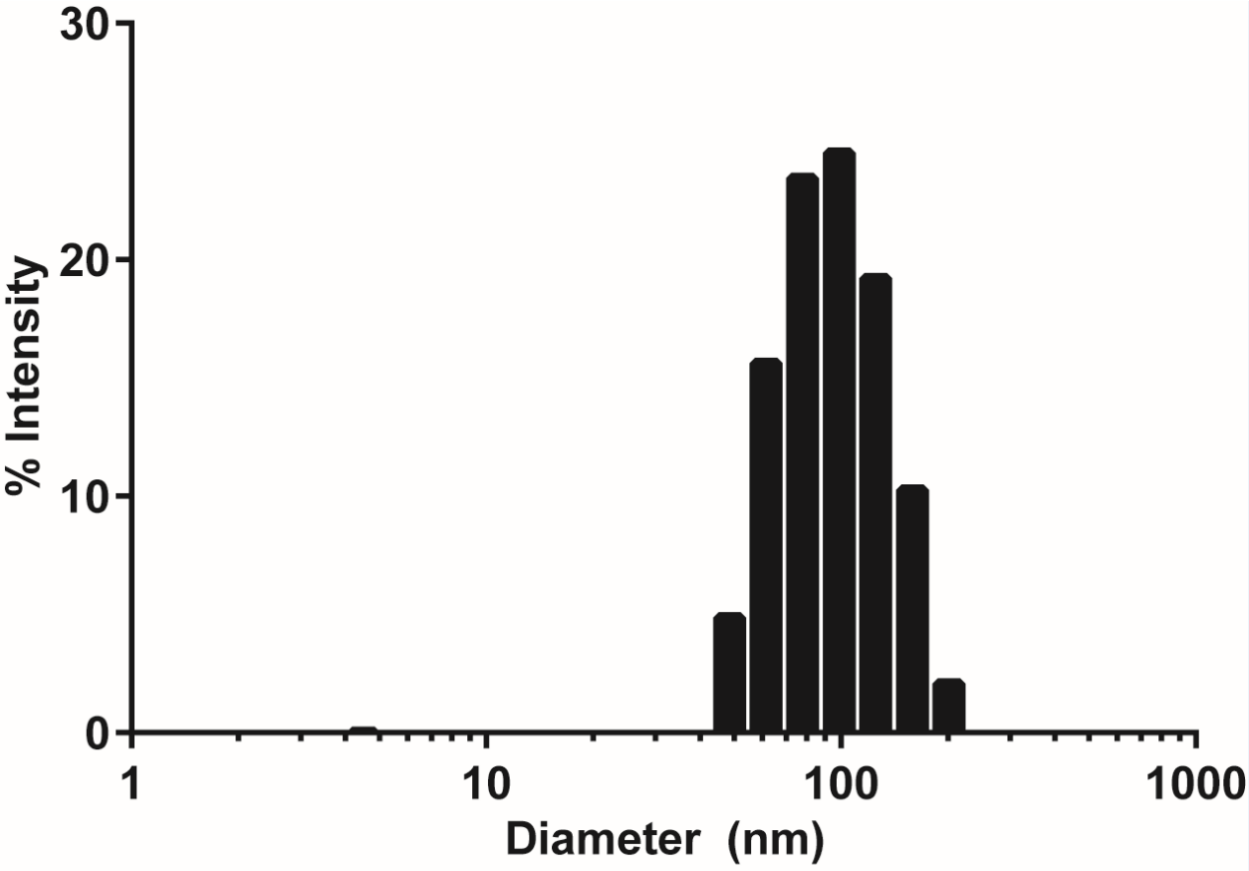
The Mass Median Aerodynamic Diameter (MMAD) of the aerosol droplet size was measured at the port of chamber using a Marple Impactor at 2 L/min sampling air flow rate.

Forced hydrolysis of TPD to TRE

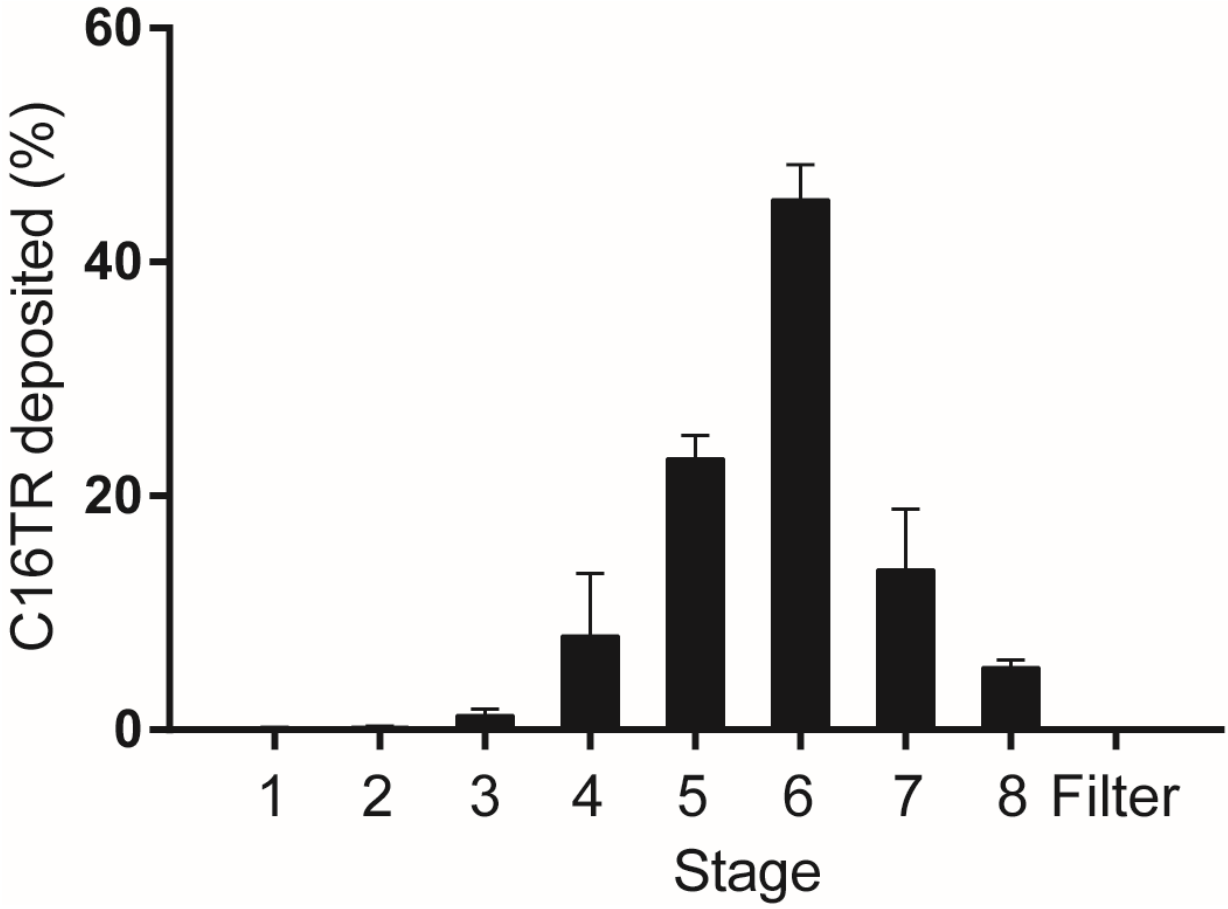
Forced degradation analyses of TPDs were performed to evaluate their initial stability and rate of hydrolysis to TRE. TPDs were prepared in water with 20% ethanol at 200 µM and incubated at 40^{0}C. TRE was quantified by HPLC after 0, 1, 2, 4, 6, and 24 hours of incubation.

Enzyme-Mediated conversion of TPD to TRE

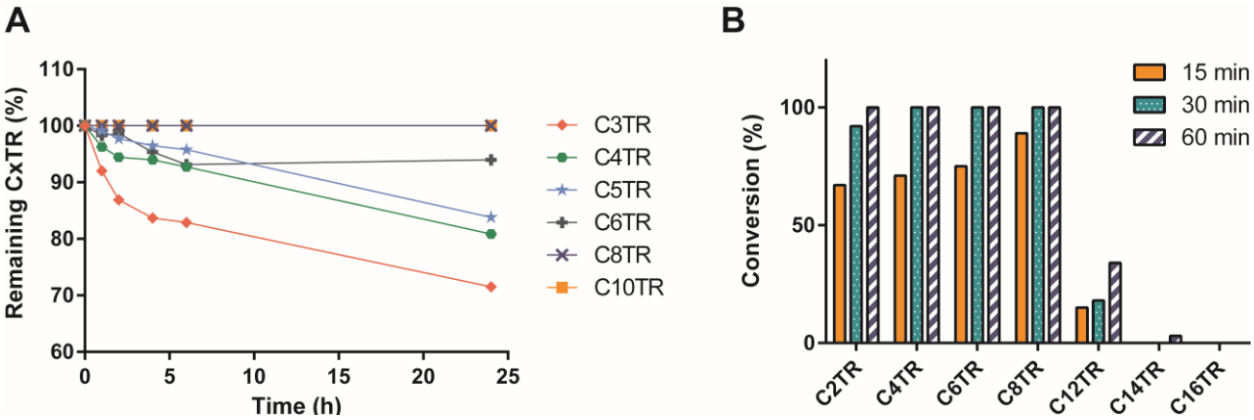
TPD (0.2 µmole) were incubated with 0.2 U of porcine esterase from Sigma (St. Louis, MO, USA) in a total volume of 500 µL PBS with 20% ethanol with a final TPD concentration of 0.4 mM. Samples were analyzed by HPLC after 0, 15, 30, and 60 min of incubation at 37 ℃.



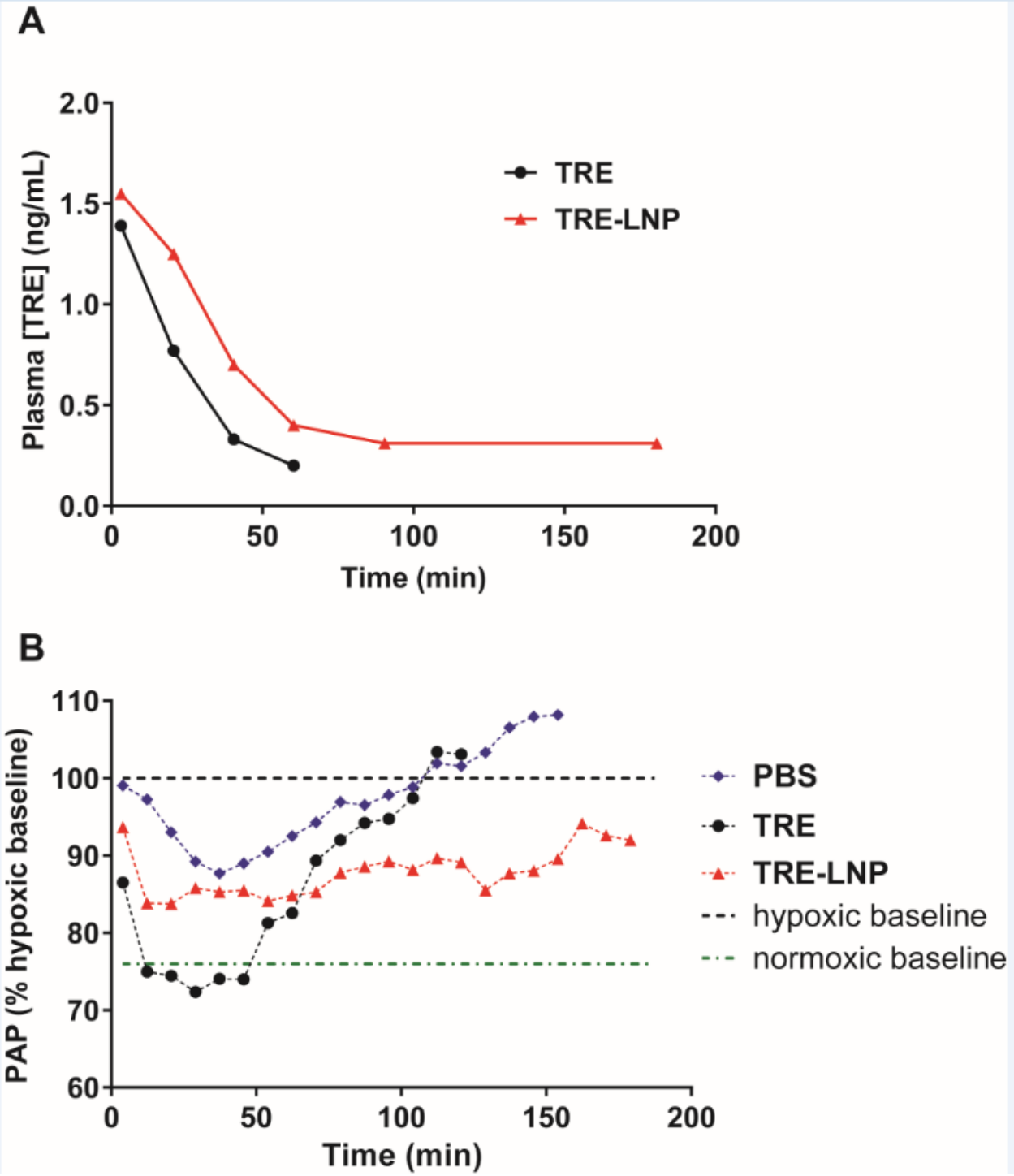
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4

Supporting Table S1: NMR and MS Characterization of TPDs

Octyl Treprostinil (C8TR): **^{1}H NMR** (500 MHz, $CDCl_3$) δ 0.88-0.92 (m, 6H), 1.18 (q, J = 11.5 Hz, 1H), 1.27-1.35 (m, 16H), 1.42-1.47 (m, 3H), 1.54-1.58 (m, 2H), 1.58-1.68 (m, 4H), 1.85-1.94 (m, 1H), 2.18-2.22 (m, 2H), 2.47 (d of d, J = 14.5 Hz, J = 6.5 Hz, 1H), 2.55 (d of d, J = 14.5 Hz, J = 6.5 Hz, 1H), 2.76 (d of d, J = 14.5 Hz, J= 6.0 Hz, 1H), 2.90 (d of d, J = 14.5 Hz, J = 6.0 Hz, 1H), 3.62 (s, 1H), 3.74-3.76 (m, 1H), 4.19 (t, J = 6.5 Hz, 2H), 4.62 (s, 2H), 6.64 (d, J = 8.0 Hz, 1H), 6.81 (d, J = 8.0 Hz, 1H), 7.07 (t, J = 8.0 Hz, 1H) ppm; **^{13}C NMR** (125 MHz, $CDCl_3$) δ 14.3 (2), 22.9 (2), 25.6, 26.0, 28.8, 29.4, 32.0, 32.1, 33.0, 35.2, 37.6, 41.5, 41.7, 52.5, 65.6, 66.2, 72.8, 77.7, 109.8, 121.7, 126.3, 128.0, 141.2, 155.1, 169.5 ppm; **HRMS** (ESI, 1:1 MeCN:H_2O): m/z = 525.3546 ($[M+Na]^+$).

Decyl Treprostinil (C10TR): **^{1}H NMR** (500 MHz, $CDCl_3$) δ 0.88-0.92 (m, 6H), 1.18 (q, J = 11.5 Hz, 1H), 1.27-1.34 (m, 20H), 1.42-1.47 (m, 3H), 1.55-1.58 (m, 2H), 1.64-1.75 (m, 4H), 1.84-1.92 (m, 1H), 2.18-2.25 (m, 2H), 2.47 (d of d, J = 14.5 Hz, J = 6.5 Hz, 1H), 2.55 (d of d, J = 14.5 Hz, J = 6.5 Hz, 1H), 2.74 (d of d, J = 14.5 Hz, J= 6.0 Hz, 1H), 2.90 (d of d, J = 14.5 Hz, J = 6.0 Hz, 1H), 3.63 (s, 1H), 3.74-3.77 (m, 1H), 4.19 (t, J = 6.5 Hz, 2H), 4.62 (s, 2H), 6.64 (d, J = 8.0 Hz, 1H), 6.82 (d, J = 8.0 Hz, 1H), 7.07 (t, J = 8.0 Hz, 1H) ppm; **^{13}C NMR** (125 MHz, $CDCl_3$) δ 14.3 (2), 22.9 (2), 25.6, 26.0, 26.2, 28.8, 28.9, 29.4, 29.5, 29.7, 32.1, 33.0, 34.0, 35.2, 37.6, 41.5, 41.7, 52.5, 65.6, 66.2, 72.8, 77.7, 109.8, 121.7, 126.3, 128.0, 141.2, 155.1, 169.5 ppm; **HRMS** (ESI, 1:1 MeCN:H_2O): m/z = 553.3858 ($[M+Na]^+$).

Dodecyl Treprostinil (C12TR): **^{1}H NMR** (500 MHz, $CDCl_3$) δ 0.86 (q, J = 7.0 Hz, 6 H), 1.15 (q, J = 10.0 Hz, 1 H), 1.24-1.32 (m, 24H), 1.38-1.46 (m, 3H), 1.50-1.54 (m, 2H), 1.59-1.64 (m, 4H), 1.81-1.88 (m, 3H), 2.13-2.26 (m, 2H), 2.42 (d of d, J = 15.0 Hz, J = 6.5 Hz, 1H), 2.52 (d of d, J = 15.0 Hz, J = 6.5 Hz, 1H), 2.73 (d of d, J = 15.0 Hz, J = 6.5 Hz, 1H), 2.86 (d of d, J = 15.0 Hz, J = 6.5 Hz, 1H), 3.58 (s, 1H), 3.68-3.73 (m, 1H), 4.16 (t, J = 7.0 Hz, 2H), 4.58 (s, 2H), 6.60 (d, J = 8.0 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 7.02, (t, J = 8.0 Hz, 1H) ppm; **^{13}C NMR** (125 MHz, $CDCl_3$) δ 14.0 (2), 22.5, 22.6, 25.3, 25.7, 25.9, 28.4, 28.6, 29.1, 29.2, 29.4 (2), 29.5, 31.8, 32.7, 33.7, 34.9, 37.3, 41.2, 41.3, 52.2, 65.2, 65.9, 72.4 109.5, 121.4, 126.0, 127.6, 140.9, 154.8, 169.2 ppm; **HRMS** (ESI, 1:1 MeCN:H_2O): m/z = 557.4221 ($[M-H]^-$).

Tetradecyl Treprostinil (C14TR): **^{1}H NMR** (500 MHz, $CDCl_3$) δ 0.90 (m, 6H), 1.14-1.19 (m, 1H), 1.27-1.35 (m, 28H), 1.40-1.57 (m, 5H), 1.62-1.67 (m, 4H), 1.84-1.91 (m, 1H) 1.94 (s, 2H), 2.15-2.28 (m, 2H), 2.46 (d of d, J = 14.5 Hz, J = 7.0 Hz, 1H), 2.54 (d of d, J = 14.5 Hz, J = 7.0 Hz, 1H), 2.75 (d of d, J = 14.5 Hz, J = 6.0 Hz, 1H), 2.89 (d of d, J = 14.5 Hz, J = 6.0 Hz, 1H), 3.61 (s, 1H), 3.71-3.76 (m, 1H), 4.19 (t, J = 7.0 Hz, 2H), 4.61 (s, 2H), 6.63 (d, J = 8.0 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 7.06 (t, J = 8.0 Hz, 1H) ppm; **^{13}C NMR** (125 MHz, $CDCl_3$) δ14.1, 14.2, 22.7, 22.7, 26.0, 29.6, 32.8, 33.8, 35.0, 37.4, 41.3, 41.4, 52.32, 65.4, 66.0, 72.6, 77.4, 109.6, 121.5, 126.1, 127.8, 141.0, 154.9, 169.3 ppm; **HRMS** (ESI, 1:1 MeCN:H_2O): m/z = 585.4536 ($[M-H]^-$).

Hexadecyl Treprostinil (C16TR): **^{1}H NMR** (500 MHz, $CDCl_3$) δ 0.87 (q, J = 7.0 Hz, 6H), 1.12-1.18 (m, 1H), 1.24-1.32 (m, 32H), 1.39-1.45 (m, 3H), 1.51-1.56 (m, 2H), 1.60-1.65 (m, 6H), 1.83-1.89 (m, 1H), 2.14-2.27 (m, 2H), 2.44 (d of d, J = 14.0 Hz, J = 7.0 Hz, 1H), 2.53 (d of d, J = 15.0 Hz, J = 6.0 Hz, 1H), 2.73 (d of d, J = 14.0 Hz, J = 7.0 Hz, 1H), 2.87 (d of d, 15.0 Hz, 6.0 Hz, 1H), 3.59 (s, 1H), 3.70-3.75 (m, 1H), 4.16 (t, J = 7.0 Hz, 2H), 4.59 (s, 2H), 6.60 (d, J = 8.0 Hz, 1H), 6.78 (d, J = 8.0 Hz, 1H), 7.03 (t, J = 8.0 Hz, 1H), ppm; **^{13}C NMR** (125 MHz, $CDCl_3$) δ 14.1, 14.2, 22.7, 25.4, 25.8, 26.0, 28.6, 28.7, 29.2, 29.4, 29.5, 29.6, 29.7, 31.9, 32.8, 33.8, 35.0, 37.5, 41.3, 41.5, 52.3, 65.4, 66.1, 72.6, 109.6, 121.5, 126.1, 127.7, 141.0, 154.9, 169.3 ppm; **HRMS** (ESI, 1:1 MeCN:H_2O): m/z = 613.4851 ($[M-H]^-$).

Supporting Table S2: Summary of Aerosol Droplet Distributions: Mass Median Aerodynamic Diameter (MMAD), Geometric Standard Deviation (GSD) and Fine Particle Fraction (FPF, size < 5 µm) of C16TR formulation.[21]

	Run 1	Run 2	Run 3	Average	SD
MMAD (µm)	**2.19**	**2.52**	**2.38**	**2.36**	**0.17**
GSD (µm)	**2.00**	**2.06**	**2.05**	**2.04**	**0.03**
FPF (%)	**88.36**	**82.92**	**84.96**	**85.4**	**2.7**