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A Dicarboxylic Fatty Acid Derivative of Paclitaxel for Albumin Assisted Drug Delivery

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Abstract

Paclitaxel is a potent chemotherapy for many cancers but it suffers from very poor solubility. Consequently the TAXOL formulation uses copious amounts of the surfactant Cremophor EL to solubilize the drug for injection resulting in severe hypersensitivity and neutropenia. In contrast to Cremophor EL, presented is a way to solubilize paclitaxel (PTX) by conjugation of a dicarboxylic fatty acid for specific binding to the ubiquitous protein, serum albumin. The conjugation chemistry was simplified to a single step using the activated anhydride form of 3-pentadecylglutaric (PDG) acid which is reactive to a variety of nucleophiles. The PDG derivative is less cytotoxic than the parent compound and was found to slowly hydrolyze to PTX (~5% over 72 h) in serum, tumor cytosol, and tumor tissue homogenate. When injected intravenously to tumor bearing mice, [³H]-PTX in the TAXOL formulation was cleared rapidly with a half-life of 7 hours. In the case of the PDG derivative of PTX, the drug is quickly distributed and approximately 20% of the injected dose remained in the vasculature experiencing a 23-h half-life. These improvements from modifying PTX with the PDG fatty acid present the opportunity for PDG to become a generic modification for the improvement of many therapeutics.

INTRODUCTION

Paclitaxel (PTX) is a mitotic spindle inhibitor primarily indicated for ovarian cancer or in combination therapy for the treatment of non-small cell lung cancer and breast cancer. With an intrinsic solubility of 10 µg/mL,¹ PTX requires dissolution in ethanol and the surfactant Cremophor EL in the commonly known product, TAXOL. The large quantities of Cremophor EL lead to neutropenia and hypersensitivity in patients thus requiring a prophylactic regimen of dexamethasone.² A second-generation formulation devoid of the surfactant uses the endogenous serum protein albumin as a vehicle. ABI-007, also known as ABRAXANE, utilizes the intrinsic binding of PTX to albumin to entrap the drug in particles of approximately 130 nm diameter after co-nebulization.² ABRAXANE showed itself superior to TAXOL in efficacy, half-life, and toxicity and is currently indicated as a second line therapy for breast cancer.^{3, 4a}

Albumin is a highly soluble protein with a vasculature concentration of 40–50 mg/mL (0.6–0.75 mM) making it the most abundant serum protein.⁵ The high vasculature concentrations of albumin are maintained due to recycling via the protective Brambell receptor (FcRn) which grants albumin and IgG 19 and 21-day half-lives in humans respectively.⁵ The use of albumin as a drug carrier for antineoplastics is not a novel concept⁶ as albumin has been

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shown to be a major nutritional staple for a variety of growing tumors.⁷ The high vascular concentrations beget high tumoral concentrations making albumin abundantly available for catabolism.^{8, 9} This tumoral accretion is believed to proceed via passive filtration and sequestration in the tumor microenvironment of leaky endothelia. This observation was the foundation for Maeda's now universal "enhanced permeability and retention" hypothesis.^{6, 10} It has recently been shown that albumin and albumin based therapies such as ABRAXANE may be more specifically sought by tumors. One mechanism of selection is through caveolae-mediated transcytosis via selective overexpression of gp60 (albionin) in the tumor endothelium. Inhibition of this protein had profound effects on the tumor accumulation of ABRAXANE.^{11, 12} Another albumin binding protein, the secreted protein acidic and rich in cysteines (SPARC), is also believed to facilitate the accumulation of albumin via increased paracellular transit due to endothelial remodeling.¹³ Increased expression of SPARC directly correlated with the efficacy of ABRAXANE showing it to be more effective than surfactant based PTX or docetaxel, even in potentially PTX resistant HER2 positive xenografts.¹⁴ SPARC has also been linked to the upregulation of matrix metalloproteases¹⁵ which is partially responsible for the high proteolytic activity observed in tumor tissues.^{16, 17} This selectivity for albumin by tumors makes albumin excellently suited as a natural drug carrier for cancer therapy.

The primary function of albumin is plasma expansion accomplished by maintaining an oncotic pressure across the vasculature with high (0.60–0.75 mM) intravascular and lower (0.30 mM) extravascular concentrations.⁵ Ancillary to expansion, albumin acts as a sponge for various metals, hydrophobic xenobiotics and fatty acids.¹⁸ Albumin was shown to have 6–7 binding sites for long-chain fatty acids with dissociation constants (K_d) in the nanomolar range.^{19, 20} These highly specific binding sites are due to hydrophobic channels in the protein which cradle the fatty acid tails. The apex of these channels, at the protein/water interface, provides lysine or arginine cations which anchor the anionic carboxylate in place via an electrostatic interaction.^{21, 22} Historically, drug-fatty acid conjugation was typically accomplished through amides or esters consequently removing the electrostatic interaction between conjugate and protein.²³ Such examples include taxoprexin²⁴ and squalenoyl-PTX.²⁵ The affinities of these conjugates for the protein are thus expected to diminish when compared to the respective unconjugated fatty acids. One exception is the myristoylated insulin LEVEMIR where the C-terminal anion of the B-chain can fortuitously bring about the gain in binding energy from the electrostatic interaction.²⁶ In the present study a new fatty acid, 3-pentadecylglutaric (PDG) acid, is presented that can be easily conjugated to nucleophiles. The product of the reaction is a fatty acid derivative that maintains an anionic carboxylate for the purpose of generating long-circulating, soluble, albumin-bound conjugates. The technology is demonstrated through a PDG-PTX conjugate (PP), but could potentially be applied to any chemical entity with a nucleophilic functionality that requires an increase in solubility, serum half-life or potentially increased tumoral delivery.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals involved in the synthesis were purchased from Sigma-Aldrich at >98% purity and used without further purification. Delipidated human serum albumin was also purchased from Sigma-Aldrich at 96% purity. All solvents were purchased from BDH and were ACS grade or better. PTX was purchased from AK Scientific (Union City, CA) and used without further purification. CT26 murine colon carcinoma cells were obtained from ATCC and grown at 37°C at 5% CO₂ in RPMI-1640 media supplemented to 10% fetal bovine serum, 1% penicillin and streptomycin. Cells were passaged twice before implantation to mice. Tritium labeled PP and PTX were synthesized by Moravek Biochemicals (Brea, CA) at a

specific activity of 11 Ci/mmol and 36 Ci/mmol respectively with ^3H -substitutions at the *ortho* positions on the benzamide ring (Fig. 2). Female BALB/c 6–8 week old mice weighing 18–20 g were acquired from NCI. Mice were allowed 1 week acclimation prior to beginning studies. All animal studies were performed under approval of the University of North Carolina Office of Animal Care and Use in accordance with the standards set forth by the Institutional Animal Care and Use Committee.

Spectroscopy

Mass spectra were obtained on an Agilent 1100-DAD-FLD Ion Trap ESI mass spectrometer in MeOH or MeCN. ^1H NMR and ^{13}C NMR spectra were recorded on an Inova 400 MHz NMR at 30°C in CDCl_3 or CD_3OD ; chemical shifts are referenced to TMS (δ 0.00 ppm) for ^1H NMR and CDCl_3 (δ 77.23 ppm) or CD_3OD (δ 49.15 ppm) for ^{13}C NMR. UV spectra were obtained with a Shimadzu UV-2401PC spectrophotometer. Scintillation counting was conducted on a Packard Tri-Carb 2900TL. Dynamic light scattering was acquired on a Malvern Zetasizer Nano ZS detecting 173° non-invasive backscatter.

Ethyl-(E)-octadec-2-enoate (1)—The intermediate was prepared following a literature procedure²⁷ (85% yield over two steps). ^1H NMR (400 MHz, CDCl_3) δ 6.96 (m, 1H, $\text{HC}=\text{CH}-\text{CH}_2$), 5.81 (d, 1H, $\text{HC}=\text{CH}-\text{CH}_2$), 4.18 (m, 2H, $\text{O}-\text{CH}_2\text{CH}_3$), 2.19 (q, 2H, γCH_2), 1.54 (s, 1H, $-\text{CH}_2-\text{CH}-\text{CH}_2$), 1.45 (t, 2H, δCH_2), 1.30–1.26 (m, 29H, $-\text{CH}_2\text{CH}_2-$, $-\text{OCH}_2\text{CH}_3$), 0.88 (t, 3H, CH_3). ESI-MS (pos, MeOH): 333.5 $[\text{M}+\text{Na}]^+$.

3-Pentadecyldiethylglutarate (2)—To 10 mL solution of anhydrous EtOH containing sodium metal (111 mg, 4.83 mmol) was added diethylmalonate (386 mg/367 μL , 2.42 mmol) and **(1)** (500 mg, 1.61 mmol) in 5 mL dry EtOH at 0°C. The solution was stirred overnight at room temperature. The solution was concentrated, diluted with water and extracted with EtOAc. The EtOAc layer was dried and evaporated to a colorless oil. To the oil in DMSO was added 1 eq of H_2O and 2 eq of NaCl at a concentration of 6 g/mL and the solution refluxed for 5 h. Dilution with water and extraction into EtOAc yielded a colorless oil. The product was purified on silica gel eluting with 5% EtOAc in petroleum ether (603 mg, 94% over two steps). ^1H NMR (400 MHz, CDCl_3) δ 4.13 (q, 4H, $-\text{OCH}_2\text{CH}_3$), 2.34 (m, 5H, $-\text{CH}_2-\text{CH}-\text{CH}_2-$), 1.33–1.24 (m, 34H, $-\text{CH}_2\text{CH}_2-$, $-\text{OCH}_2\text{CH}_3$), 0.88 (t, 3H, CH_3). ^{13}C NMR (100 MHz, CDCl_3) δ 172.8, 60.4, 38.9, 34.2, 32.4, 32.1, 29.8, 26.8, 22.9, 14.4, 14.3. ESI-MS (pos, MeOH) m/z 399.7 $[\text{M}+\text{H}]^+$, 421.8 $[\text{M}+\text{Na}]^+$, 437.8 $[\text{M}+\text{K}]^+$.

3-Pentadecylglutaric acid (3)—To 15 mL of MeOH containing **2** (1.06 g, 2.66 mmol) was added 30 mL 3 N KOH. The mixture was heated to a reflux with stirring for 3 h causing the suspension to become clear. The solution was cooled to ambient temperature, acidified to pH 1 with HCl and extracted with methyl *t*-butyl ether. The organic layer was dried and concentrated and the product collected as a white solid (900 mg, 99%). The compound was pure by TLC 10% Me_2CO in petroleum ether. ^1H NMR (400 MHz, CD_3OD) δ 2.33–2.27 (m, 5H, $\text{CH}_2-\text{CH}-\text{CH}_2$), 1.37–1.29 (m, 28H, $-\text{CH}_2\text{CH}_2-$), 0.90 (t, 3H, $-\text{CH}_3$). ^{13}C NMR (100 MHz, CD_3OD) δ 176.7, 39.6, 35.2, 33.5, 33.3, 30.9, 27.8, 23.9, 14.6. ESI-MS (neg, MeOH) m/z 341.7 $[\text{M}-\text{H}]^-$.

3-pentadecylglutaric anhydride (4)—Intermediate **3** (900 mg, 2.63 mmol) was suspended in 3 mL EtOAc and heated gently until dissolved. To the solution was added trifluoroacetic anhydride (773 mg/0.513 mL, 3.68 mmol) and the solution was heated to 37°C with shaking for 2 h. After evaporation the solid was recrystallized from pentane. Filtration of the solid and drying under vacuum produces the product as a fluffy white powder in quantitative yield (853 mg, 100%). ^1H NMR (400 MHz, CDCl_3) δ 2.89 (dd, 2H, $\text{CH}_2-\text{CH}-\text{CH}_2$), 2.43 (dd, 2H, $\text{CH}_2-\text{CH}-\text{CH}_2$), 2.19–2.12 (m, 1H, $\text{CH}_2-\text{CH}-\text{CH}_2$), 1.40–1.26

(m, 28H, $-\text{CH}_2\text{CH}_2-$), 0.88 (t, 3H, $-\text{CH}_3$). ^{13}C NMR (100 MHz, CDCl_3) δ 167.1, 36.3, 34.7, 32.1, 29.8, 28.9, 26.5, 22.9, 14.3. ESI-MS (pos, MeCN) m/z 325.4 $[\text{M}+\text{H}]^+$, 347.6 $[\text{M}+\text{Na}]^+$.

Paclitaxel-2'-O-3-pentadecylhemiglutarate (5; PP)—Intermediate **4** (21 mg, 64.5 μmol), PTX (50 mg, 58.6 μmol), and *N,N*-dimethylaminopyridine (DMAP) (0.7 mg, 5.86 μmol) were dissolved in 0.8 mL dry pyridine and the reaction mixture stirred overnight at room temperature. After evaporation the solid was taken up in dichloromethane (DCM) and washed with 0.1 N HCl. The DCM was dried, filtered and concentrated. The solid was adsorbed onto silica and eluted with (1:1) EtOAc in petroleum ether supplemented with 1% AcOH. The product was recovered as a white solid (58.4 mg, 85%). ^1H NMR (400 MHz, CDCl_3) δ 8.12 (d, 2H, ArH), 7.75 (d, 2H, ArH), 7.62-7.24 (m, 11H, ArH), 6.29 (s, 1H, C10), 6.24 (t, 1H, C-13), 5.99 (dd, 1H, C-3'), 5.68 (d, 1H, C-2), 5.50 (d, 1H, C-2'), 4.97 (d, 1H, C-5), 4.40 (dd, 1H, C-7), 4.29 (d, 1H, C-20), 4.20 (d, 1H, C-20), 3.80 (d, 1H, C-3), 2.56-2.50 (m, 1H, $-\text{CH}_2-\text{CH}-\text{CH}_2-$), 2.45 (s, 3H, OAc), 2.40-2.28 (m, 3H, $-\text{CH}_2-\text{CH}-\text{CH}_2-$, C-6 α), 2.24-2.13 (m, 7H, OAc, C-14, $-\text{CH}_2-\text{CH}-\text{CH}_2-$), 2.00-1.85 (m, 4H, C-18, C-6 β), 1.66 (s, 3H, C-19), 1.26-1.19 (m, 31H, $-\text{CH}_2\text{CH}_3$, C-17), 1.13 (s, 3H, C-16), 0.88 (t, 3H, $-\text{CH}_2\text{CH}_3$). ^{13}C NMR (100 MHz, CDCl_3) δ 204.0, 176.7, 171.9, 171.5, 170.2, 168.3, 167.7, 167.2, 142.9, 137.1, 133.8, 133.0, 132.2, 130.5, 129.0, 127.1, 84.7, 81.3, 79.2, 75.8, 75.3, 74.4, 72.2, 58.7, 53.1, 45.8, 43.4, 38.0, 35.7, 34.1, 32.2, 29.9, 29.7, 27.1, 22.9, 22.3, 21.0, 15.0, 14.3, 9.8. ESI-MS (pos, MeOH) m/z 1201.1 $[\text{M}+\text{Na}]^+$.

Cytotoxicity of PP

The cytotoxicity of PP and PTX was determined by the NCI as part of the NCI-60 DTP Human Tumor Cell Line Screen.²⁸ Briefly, various human cancer cell lines are plated at 5,000–40,000 cells/well and treated with five concentrations of PP or PTX from 10 nM to 100 μM . The effect on cell growth is compared to cell lines fixed at time 0 and the untreated control via the sulforhodamine B test. Dose response curves are then generated across 60 cell lines and the 50% growth inhibition (GI50), total growth inhibition (TGI) and 50% lethal concentration (LC50) values are determined for each cell line.

Biochemical Stability of PP

Murine-derived CT26 cells were grown in a T-75 flask to confluency ($\sim 4 \times 10^6$ cells). The cells were then washed twice with Hank's balanced salt solution (HBSS), scraped off the flask with a cell scraper and suspended in 5 mL HBSS. The suspension was centrifuged at 600 x g for 5 min at 4°C. The pellet was homogenized in 0.5 mL fresh HBSS on ice using a Potter-Elvehjem homogenizer attached to an Hitachi 12-Volt 3/8" cordless peak lithium ion micro driver drill at 1300 rpm while continuously plunging for 15 minutes. After centrifugation at 6000 x g, the supernatant was diluted to 1 mL and stored in 100- μL aliquots at -18°C until use. Prior to use, the aliquots were diluted another 2-fold with HBSS.

Tumor tissue homogenate was prepared by inoculating 5×10^5 CT26 cells on the right flank of an 8-week old female BALB/c mouse, and the tumor allowed to grow to $\sim 1000 \text{ mm}^3$. At this point, the mouse was anesthetized with 0.1 mL 100 mg/mL intraperitoneal ketamine with subsequent cervical dislocation. The tumor mass was excised, washed with phosphate-buffered saline (PBS) and blotted dry. The tumor was weighed and diluted with 2 volumes of PBS. The suspension was coarsely homogenized on ice using a POWERGEN 700 tissue homogenizer (Fisher Scientific) for 5 minutes until no lumps of tissue remained. The tissue homogenate was mixed thoroughly, separated into 1-mL aliquots and stored at -18°C until use.

For serum preparation, 8 female 8-week old BALB/c mice were anesthetized with ketamine until unresponsive to toe pinching. The mice were then exsanguinated by cardiac puncture

with subsequent cervical dislocation. The whole blood was pooled and stored at 4°C overnight. The fibrin clot was pelleted by centrifuging the suspension at 6000 x g at 4°C and the supernatant removed and separated into 500-μL aliquots.

The extraction efficiencies of both PP and PTX were determined in duplicate using the individual milieu that was spiked with radiolabeled stock solution of either [³H]-PP or [³H]-PTX. Aliquots of the stock were weighed out and analyzed by liquid scintillation counting (LSC) to determine the concentration. Next the matrices were extracted with 1 volume of 1 N HCl and 8 volumes of MeCN. The samples were centrifuged at 16.1 x 1000g for 5 min and the supernatants quantitated by LSC. Efficiencies were calculated as a ratio of concentration found from the extraction over the initial concentration in the matrix.

The disappearance of PP and concomitant appearance of PTX in the test milieus were monitored by an LC/MS procedure. Standard curves were generated by combining PP (160 to 1 μM) and PTX (1 to 160 μM) stock solutions in EtOH with aliquots of the test milieu (serum, cell, or tissue homogenate) at a 1:10 (v/v) ratio respectively. The mixtures were then diluted with equal volumes of 1 N HCl and precipitated with 8 volumes of MeCN containing 500 nM docetaxel as internal standard. The stability of the PP was carried out at 16 μM in duplicate at 37°C while shaken continuously. At given times, 20-μL aliquots were mixed with 20 μL 1N HCl followed by 160 μL MeCN containing 500 nM docetaxel. The precipitate was pelleted at 800 x g for 2 minutes and the supernatant removed for analysis on an Aquasil C18 column (2.1 x 50 mm) with 5 μm particles and 80 Å pore size using 20-μL injections.

The eluent was a binary gradient containing 0.1% formic acid in water (A) and 0.1% formic acid in MeOH (B). The gradient began with 1 min of 30% B followed by a linear increase over 2 mins to 95% B. The eluent was then held constant at 95% B for 15 sec followed by a 30 sec linear decrease down to 30% B and 30 sec re-equilibration at 30% B. The flow rate and sample volume were 0.75 mL/min and 100 μL, respectively. Analysis was by an API 4000 Triple Quadrupole LC/MS/MS mass spec (AB Sciex Instruments) scanning for parent to daughter transitions of 854-525 m/z (PTX) and 854-525 m/z (PP) in negative mode. The 1178-854 transition for PP was inconsistent and thus the daughter PTX and its subsequent daughter fragment were used for analysis for both the standard curve and stability study. Concentrations were determined using peak area per the standard curves.

Formulation of PP with Albumin and PTX as TAXOL

The concentration of PP in a stock solution in *t*-BuOH was confirmed via UV absorption in EtOH ($\epsilon_{228}=30,593 \text{ M}^{-1}\text{cm}^{-1}$, EtOH). The PP stock was then added to a 4 or 5% (w/v) delipidated HSA in water at a 1:10 final volume ratio of EtOH:HSA ($\epsilon_{278}=39,800 \text{ M}^{-1}\text{cm}^{-1}$, water), (29) which immediately caused turbidity due to precipitation of PP. The sample was shell-frozen and lyophilized over 24 h. The lyophilized powder was then reconstituted in 0.1 M phosphate buffer at pH 7.4 and filtered through a 0.22 μm PVDF filter (Millipore Billerica, MA). If the stock contained [³H]-PP, it was quantified via liquid scintillation counting (LSC). If the stock is not radiolabeled, it is extracted by diluting with one volume of 1 N HCl and 8 volumes of MeCN then added a quantity sufficient of EtOH to make the volume 5 mL in a volumetric flask for UV/Vis analysis. The loading efficiency of PP varied depending on such processing parameters as injection rate, stirring dynamics, concentration of albumin and ionic strength with a maximum loading of about 50% for higher doses. TAXOL was prepared by dissolving PTX at 7.5 mg/mL in a 1:1 (v/v) mixture of EtOH:Cremophor EL. The drug concentration was confirmed by diluting with EtOH and analyzing by UV absorption ($\epsilon_{228}=29.800 \text{ M}^{-1}\text{cm}^{-1}$, EtOH). The organic stock was diluted 5-fold with a phosphate buffer to produce a concentration of about 1.5 mg/mL immediately prior to injection.

Dialysis of PP-HSA Complex

To determine whether the PP-HSA complex would dissociate over time, the complex was dialyzed against delipidated human serum albumin in phosphate buffer to create sink conditions. Dialysis was conducted across a Spectra Por 12–14 kDa MWCO regenerated cellulose dialysis membrane with 500 μ L fiberglass compartments in a custom-made dialysis block. A 0.1 M phosphate buffer pH 7.4 containing 0.01% NaN_3 was diluted 10:1 (v/v) with EtOH. This EtOH-containing buffer was saturated by shaking with solid [^3H]-PP for 24 h at 37°C then cooled to ambient temperature and filtered through a 0.22 μ m PVDF filter. Albumin was dissolved in phosphate buffer without EtOH at concentrations of 40 mg/mL and 10 mg/mL as confirmed by UV. Finally a stock solution of [^3H]-PP in EtOH was made to a concentration of 15 mg/ 8.63×10^7 dpm/mL. The PP stock was added to the albumin stocks in a 1:10 (v/v) ratio and the stocks were filtered through a 0.22 μ m PVDF filter. These stocks were analyzed by LSC for PP content and UV for HSA content.

The saturated solution of [^3H]-PP in 0.1 M phosphate buffer pH 7.4 was dialyzed against blank phosphate buffer for 5 h with sampling of both compartments every hour to determine how quickly free PP could establish equilibrium. The albumin solutions loaded with PP were sampled after shaking for 24 h at 37°C and the compartment replenished with fresh aliquots of the initial contents. After another 24 h of shaking at 37°C, all contents were analyzed. Analysis involved weighing 200 μ L aliquots of the sample into scintillation vials and adding 5 mL Ultima Gold scintillation cocktail followed by LSC.

Pharmacokinetics/Biodistribution of [^3H]-PP-HSA and [^3H]-TAXOL

Female, 6–8 week old BALB/c mice were administered 5×10^5 CT26 murine colon carcinoma cells in 0.1 mL HBSS on the right flank. When the tumors were $\sim 1000 \text{ mm}^3$, the mice received the radiolabeled dose as formulated in 4% HSA in 0.1 M phosphate buffer (0.08 mg PP equiv to 0.058 mg PTX/ 1.75×10^7 dpm/0.1 mL) or as TAXOL (0.147 mg PTX/ 2.22×10^6 dpm/0.1 mL) via the tail vein. Approximately 5 minutes prior to the timepoint, the mice were injected *i.p.* with 0.1 mL 100 mg/mL ketamine. Once unresponsive to toe pinching, the mice were exsanguinated via cardiac puncture with subsequent cervical dislocation. The whole blood was quickly weighed as 200 μ L aliquots in triplicate. The target organs were harvested, washed with PBS, blotted dry and small (~ 100 mg) pieces excised, weighed and stored in scintillation vials in duplicate. The target organs include the liver, lungs, heart, kidneys, spleen, tumor and injection site on the tail.

To the whole blood aliquots was added 1 mL of Solvable^R tissue solubilizer. The sample was incubated for 1 hour at 55°C turning brownish-green. To this was added 100 μ L of 0.1 M ethylenediaminetetraacetic acid disodium followed by 300 μ L 30% H_2O_2 in 100 μ L aliquots. The reaction can be quite vigorous and was allowed to continue at room temperature until the foaming subsided. The samples were then heated to 55°C for an additional hour yielding clear to slightly yellow solutions. The samples were cooled to ambient and 15 mL Ultima Gold scintillation cocktail was added and mixed thoroughly. The samples were rested in the liquid scintillation counter for 1 h to adapt to light and temperature prior to counting.

To the organs was added 1 mL of Solvable[®] followed by heating to 55°C for 2 h. Once dissolved (yellow to slightly yellow) the samples were cooled to ambient and 200 μ L of 30% H_2O_2 was added in 100 μ L aliquots. The samples were then heated to 55°C again for 30 min. The samples were cooled to ambient and 15 mL Ultima Gold scintillation cocktail was added and thoroughly mixed. The samples were rested in the LSC for 1 h to adapt to light and temperature prior to counting.

Aliquots of PP stock were weighed in triplicate and analyzed via liquid scintillation counting. A tumor bearing mouse was then anesthetized with 0.1 mL 100 mg/mL ketamine i.p. and exsanguinated via cardiac puncture. The mouse was sacrificed and the target organs harvested. Aliquots of 175 μ L, 200 μ L and 225 μ L of blood were weighed and spiked with a weighed amount of PP stock ($\sim 1 \times 10^4$ dpm). Additionally, ~ 75 , 100 and 125 mg samples of organs were washed, blotted dry and weighed then spiked with a weighed amount of PP stock ($\sim 1 \times 10^4$ dpm). The samples were then processed in the same fashion as the unknown samples. The counting efficiency was calculated as the ratio of dpm in the organ sample / calculated PP stock dpm $\times 100$. The efficiencies were plotted as a function of organ or blood mass and fitted with a linear regression. The calculated efficiency was then used to correct the counts for the unknown samples based on blood volume or organ mass.

RESULTS

Chemistry

The synthesis of 3-alkylglutaric anhydrides has already been demonstrated.³⁰ This synthetic method was shortened and modified (Fig. 1). Hexadecanol was oxidized to palmitaldehyde using PCC and subsequently subjected to Wittig olefination using the ylide (carboethoxymethylene)triphenylphosphorane.²⁷ The α,β -unsaturated ethyl ester can then be used as a Michael acceptor for diethyl sodiomalonate at room temperature.³¹ The resultant triester is decarboxylated and saponified to the 3-alkylglutaric acid using KOH; NaOH resulted in incomplete saponification.³¹ Dehydration of the diacid can efficiently be affected with trifluoroacetic anhydride in EtOAc at 37°C.³² PDG anhydride itself is reactive to amines and alcohols thus it could react with PTX at three positions. PTX is known to be reactive to glutaric anhydride at the 2'-OH position at room temperature. The 7-OH is only reactive under heated conditions and the 1-OH is unreactive.^{33, 34} Mixing PDG anhydride and PTX in pyridine with a catalytic amount of DMAP allows complete reaction at room temperature in 24 h (Fig. 2).

Cytotoxicity *in vitro*

The IG_{50} , TGI and LC_{50} were determined for PP and PTX via the NCI-60 DTP Human Tumor Cell Line Screen. The results for all three values consistently showed a superior cytotoxicity of PTX to PP by at least an order of magnitude and up to several orders; 0.2–3 μ M for PP and 0.02–1 μ M for PTX. For simplicity, representative data from the TGI data is presented (Table 1). Due to the large number of cell lines, only two were selected from each cancer type which demonstrated the greatest toxicity for PTX and PP.

Biochemical Stability

Prior to the experiment, stock solutions of [3 H]-PP and [3 H]-PTX in EtOH were introduced to various milieu at a 1:10 (v/v) ratio. Subsequent to incubation for a given period of time, samples were taken and extracted with 1 volume of 1 N HCl and 8 volumes of MeCN, vortexed and pelleted by centrifugation. For undiluted murine serum, PP and PTX were quantitatively extracted. For CT26 cytosolic fraction, PP was extracted at 97% efficiency and PTX at 91%. For CT26 tissue homogenate, PP was extracted at 90% efficiency and PTX at 94% efficiency. Results of the stability study are presented as an average of duplicates and results from LC/MS were corrected for extraction efficiency. The stability of PP in serum, tumor cytosol and tumor tissue homogenate was determined as a function of time (Fig. 3). The PP is stable in this particular concentration of cell homogenate attaining under 1% conversion to PTX after 36 h. The PP is similarly stable in serum attaining about 1.5% conversion to PTX within 72 h. Under the experimental conditions, the PP was also stable in the tumor tissue homogenate only attaining 5.5% conversion to PTX after 72 h.

Pharmaceutics

PTX is very poorly soluble in water ($\sim 10 \mu\text{g/mL}$)¹ and adding a fatty acid to this molecule only exacerbates the issue. The solubility of PP was calculated using [^3H]-PP to be $0.36 \mu\text{g/mL}$ ($0.26 \mu\text{g/mL}$ PTX) in 0.1 M phosphate buffer at pH 7.4, room temperature. In the presence of HSA, the solubility of PP should increase dramatically to a theoretical maximum of the number of fatty acid binding sites multiplied by the concentration of HSA. This would be a solubility of about 3.6 mM (3.07 mg/mL with respect to PTX) conservatively assuming a 40 mg/mL physiological solution of albumin and 6 fatty acid binding sites. After two weeks of equilibration, the concentration of PP with respect to PTX ($54 \mu\text{g/mL}$) was substantially less than the theoretical maximum (*data not shown*).

To improve solubility, PP was dissolved in a water miscible co-solvent, *t*-BuOH, and introduced to aqueous albumin at a 1:10 (v/v) ratio. Albumin was found to be structurally stable to *t*-BuOH at this concentration via circular dichroism analysis (*data not shown*) and no aggregation was observed by DLS (Supporting Information 1). This is not surprising as other small alcohols at this concentration do not perturb its secondary or tertiary structure.³⁵ Distilled water was chosen as a solvent as electrolytes tend to salt-out PP leading to precipitation. After loading, the suspension was shell-frozen and lyophilized to a cake which was reconstituted in an isotonic solution. Filtration of the suspension yields a clear blue solution, indicative of Tyndall scattering. Analysis by dynamic light scattering showed aggregates of 120 nm diameter (Supporting Information 1). Formulation of unmodified PTX via this same procedure also led to a blue solution of PTX-HSA aggregates. To test if this phenomenon was PTX specific two water soluble conjugates were synthesized using fluoresceinamine (FLA) attached to a short tetraethyleneglycol (miniPEG) linker as a drug surrogate. The FLA-miniPEG was attached to either PDG or stearic acid (SA) such that the only difference between the two conjugates was the presence of the carboxylate anion (Supporting Information 2). The PDG-FLA-miniPEG and SA-FLA-miniPEG conjugated fatty acids as well as the corresponding unconjugated fatty acids were then introduced to albumin and analyzed by DLS with no observable aggregation (Supporting Information 1).

The extremely poor aqueous solubility of PP precluded determination of the dissociation constant via conventional methods such as isothermal titration calorimetry or equilibrium dialysis. Instead, the stability of the PP-HSA complex was examined. Dialysis of a PP solution without HSA across a $12\text{--}14 \text{ kDa}$ MWCO membrane over 5 h showed a rapid equilibrium could be attained within this time period. In contrast, dialysis of the PP-HSA complex against comparable concentrations of delipidated HSA, which should act as a sink for free PP, showed only about 3% of PP was released from the complex over 2 days at 37°C for both 4% and 1% concentrations of HSA (Table 2). To determine the effect of the anionic charge from the PDG conjugates, the water soluble FLA conjugates were introduced to albumin and analyzed by isothermal titration calorimetry. Initial results suggest the carboxylate from PDG does bring about a 3.5-fold increase in binding affinity, 2-fold decrease in enthalpy and about a 2-fold decrease in entropy (Supporting Information 3).

Pharmacokinetics/Biodistribution

The PP formulation shows a rapid distribution (Fig. 4) from the vasculature over 6 h accounting for a 10-fold dilution in serum concentrations. The concentration then rebounds to about 20% of the initial concentration with a secondary peak that is within the quantified error. This concentration is then cleared very slowly with a half-life approaching 24 h . Biodistribution analysis shows accumulation primarily in the liver and spleen (Fig. 5). The concentration in these organs also decreases slowly mirroring the clearance from the serum allowing quantitation over 72 h . The tumor rapidly accumulates 1–2% of the dose/g tissue which remains constant through 72 h .

The PTX from the TAXOL formulation shows a similarly rapid distribution (Fig. 4). No rebound is observed and the terminal half-life of the formulation was estimated to be 7 h. Biodistribution analysis shows accumulation primarily in the liver and kidneys (Fig. 5), which eliminate PTX quickly from the system such that the lower limit of detection is reached after 12 h. The tumor shows a comparable uptake of about 3% of the dose/g tissue, however concentrations approach the lower limit of detection by 6 h.

The serum pharmacokinetics were analyzed using non-compartmental analysis. The major pharmacokinetic parameters were extracted and presented in Table 2. The PP formulation shows a 3.5-fold increased serum half-life due to a lower systemic clearance and volume of distribution. Consequently, while at only half the dose of TAXOL, PP shows a higher C_0 , area under the curve (AUC) and mean residence time (MRT) in the system.

DISCUSSION

Formulation of PP-HSA Aggregates

The purpose of synthesizing the PP conjugate was to improve the solubility and pharmacokinetics of PTX by utilizing the endogenous serum protein, albumin. Loading of albumin with PP as a buffered suspension was exceedingly slow taking 2 weeks to reach only 54 $\mu\text{g/mL}$ with respect to PTX. This is in contrast to the expected 3 mg/mL at saturation of 6 binding sites of 4% HSA. The slow binding could be a result of rate-limited dissolution. To facilitate the kinetics of binding, PP was dissolved in *t*-BuOH prior to mixing with a solution of 4% HSA in water. Much of the PP precipitates but this process can result in much higher concentrations of bound PP; adding 12 eq of PP allowed full saturation of 6 binding sites of HSA. After loading the solvents are subsequently sublimed by lyophilization and the cake reconstituted in an isotonic solution suitable for injection.

The PP-HSA complex spontaneously formed 120 nm particles which remain after lyophilization and reconstitution. Once the PP forms a complex with HSA, it is exceedingly difficult to dissociate; some failed attempts include surfactants with acyl chains (tweens and SDS), high concentrations of urea, high concentrations of water miscible and immiscible organic solvents, TCA precipitation. The only reliable way to dissociate the complex was to acidify the complex to pH 1 and precipitate with EtOH or MeCN. The acidification step causes albumin to unfold from the normal heart shaped N state to the fully expanded E state, potentially exposing all the fatty acid binding pockets.³⁶ The acidification also quenches the anion from the PP conjugate making it very water insoluble but highly organic soluble. The addition of EtOH or MeCN then precipitates the protein but dissolves the PP.

The structural details of the particles are not known. Based on published docking studies from PTX and HSA, it is hypothesized the PTX moiety of the PP on the surface of albumin can interact with other PTX moieties via Van der Waals interactions or bind other molecules of albumin through PTX-binding sites.³⁷ Formulation of PTX without the PDG moiety in this manner saturates at 0.5 eq PTX/HSA with formation of 100 nm particles as confirmed by dynamic light scattering (Supporting Information 1). This finding suggests that particle formation is a PTX-specific phenomenon, as also found with ABRAXANE. This is further supported by the lack of aggregation of albumin upon binding PDG-FLA-miniPEG, SA-FLA-miniPEG, PDG acid or SA. Interestingly ABRAXANE allows solubilization of 10 equivalents of PTX/HSA compared to a maximum of 0.5 by simple mixing. Formulation as ABRAXANE would thus allow higher concentrations of PTX to be solubilized compared to PP which only has 6 potential binding sites for the fatty acid.

Cytotoxicity of PP

The PP conjugate is less cytotoxic than PTX towards a variety of human cancer cell lines when tested *in vitro* (Table 1). The mechanism of action of PTX is to bind and prevent microtubulin assembly, however, numerous PTX derivatives have been shown to relinquish this binding capacity yet retain varying degrees of cytotoxicity.^{25, 38} This appears to be regiospecific as succinate esters on the 2'-OH or 7-OH also abolish microtubule binding³³ whereas the 1-OH succinate ester retains it.³⁹ The PP conjugate was shown to be highly stable over 72 h in a variety of milieu including undiluted murine serum, CT26 cellular cytosol, and tumor tissue homogenate. The short time scale of 4 h for the *in vitro* cytotoxicity assay precludes the more active PTX from forming suggesting the PDG moiety likely affects microtubule bundling.

Pharmacokinetics/Biodistribution

Ideally albumin-bound drugs will remain invisible to the liver and reticuloendothelial system, as is the case with IgG, due to the expression of FcRn on monocytes.⁴⁰ The release mechanism for bound drugs would then be dilution-driven dissociation or release due to digestion of the carrier albumin. Dissociation from the protein is expected to be slowed due to the increased binding affinity imparted by the anionic charge of the PDG fatty acid. A dialysis experiment aimed at assessing the off-rate of PP from albumin shows it is very slow, even when sink conditions are created by dialyzing against similar concentrations of delipidated albumin (Table 2). This is not diffusion rate limited as free PP was shown to equilibrate across the membrane within hours with minimal membrane binding. Thus, spontaneous dissociation from the intact protein is unlikely to be a major release mechanism for PP *in vivo*.

Since the PP conjugate is utilizing albumin's natural utility as a fatty acid carrier but is not subject to rapid dissociation, albumin is expected to impart its physiological profile to the conjugate. Upon bolus intravenous injection of unmodified albumin a characteristic two-phase profile is observed.⁴¹ The initial phase shows a 60% removal of the dosed albumin representing rapid distribution throughout all the extravascular tissues and fluids. The slope of this phase is used to calculate the transcapillary escape rate (TER) for albumin which is estimated to be ~4–5%/h in humans. This is not irreversible clearance from the body as the re-equilibration of the albumin back into the vasculature via the lymphatic system through the thoracic duct gives rise to the protracted half-life of albumin. The second phase observed in the albumin profile is metabolic clearance and the slope is used to estimate the fractional degradation rate (FDR) of albumin, ~4%/day in humans. This profile was also observed with albumin based therapies such as ABRAXANE⁴² as well as covalent linkages to albumin such as through the single free cysteine using a maleimide linker.⁴³

This too is what was observed with the PP-HSA complex, although the secondary peak has not been seen with unmodified albumin before. This may not be a real secondary peak as the pharmacokinetic data has been repeated twice using a TAXOTERE-like formulation of PP in which the calculated pharmacokinetic parameters are very similar but no secondary peak is observed (Supporting Information 4). The observed half-life of 23 h for the PP conjugate agrees with recent data suggesting human serum albumin is not protected by murine FcRn⁴⁴ leading to a half-life approaching a day in mice. This is also comparable to the half-life of ABRAXANE which was found to be 17.1 h; in the same study TAXOL was found to have a half-life of 4.0 h.⁴⁵ While this may seem inconsequential, it is important to mention the half-life of albumin increases proportionally with size.⁴⁶ If the complex mimics the half-life of albumin in humans, as it does in mice, a 19-day half-life would be predicted. The half-life could then be modulated by controlling the affinity of the conjugate for albumin simply by reducing the alkyl length of the initial alcohol (Fig. 1).

The volume of distribution, V_d , estimates the amount of drug in the tissues as compared to the amount of drug in the blood. Larger V_d values are common for lipophilic drugs suggesting they tend to permeate through the tissues of the body readily with little remaining in the blood. Lower volumes of distribution reflect hydrophilic or macromolecular drugs that do not readily permeate the endothelium or lipophilic drugs that are highly protein bound causing retention in the vasculature. The apparent V_d for PTX from the TAXOL formulation (120 mL) was much higher than from the PP formulation (36 mL) suggesting PTX from TAXOL permeates the tissues more readily. While both molecules are highly lipophilic and bind albumin, the substantial decrease in V_d from TAXOL to PP is indicative of a much stronger binding of PP with albumin than TAXOL. Thus, PTX is expected to dissociate from albumin more readily than PP in various tissues, particularly the liver, facilitating rapid clearance. In fact, the PTX showed a 12-fold increase in clearance over PP. The PP formulation shows a 2-fold higher initial concentration and greater AUC compared to the TAXOL formulation despite being half the dose; again due to the large V_d for TAXOL. The longer retention within the vasculature creates the potential for more drug to be exposed to the tumor due to multiple passes through the tumor vasculature with about a 4.5-fold increase in mean residence time (Table 3).

Biodistribution analysis of the PP conjugate is similar to unmodified albumin demonstrating uptake throughout all major viscera but primarily the liver. In the case of unmodified albumin, the liver is a primary destination due to the large pool of blood but it is not a major site of degradation.⁴¹ The biodistribution data (Fig. 5) shows the PP is not rapidly cleared from any of the major organs and in fact the concentrations mimic the concentration in serum, including the secondary peak. This suggests the PP is not being deposited or rapidly degraded in any major tissue as is the case with albumin. No specific analysis was conducted *in vivo* to determine if the radiolabel in the PP experiment is from intact PP or PTX, however it is likely intact PP because the pharmacokinetics are much more similar to that of albumin bound formulations, ABRAXANE and maleimide based drugs, suggesting the PP-HSA complex is very tight. Additionally when PP was formulated like TAXOTERE, the docetaxel equivalent of TAXOL, the pharmacokinetics (Supporting Information 4) and biodistribution (Supporting Information 5) were very similar to PP-HSA. If the PP is hydrolyzing to PTX, which binds albumin weakly, then the kinetics should be more similar to that of the TAXOL formulation. The TAXOL formulation shows rapid equilibration into the liver, and rapid elimination from the liver. The tumor uptake is low for both formulations at 1–3% of the injected dose/g tissue, but like the serum concentrations of PP the tumor concentrations are also sustained over 3 days. Conversely for the TAXOL formulation, the serum and tumor concentrations of PTX decrease quickly.

Submicron particles, including albumin, do not diffuse far into high-pressure tumors.⁴⁷ In order for the PP-HSA complex to be cytotoxic, the albumin must be degraded in the tumor environment releasing PP which can then diffuse to the vicinal tumor cells. PP can then be activated by hydrolysis to PTX. From the literature, albumin digestion in the tumor vicinity is expected to be rampant.¹⁷ Conversely the hydrolysis of PP to PTX (Fig. 3) appears to be slow in both CT26 tumor cell cytosol as well as the tumor tissue homogenate. Because of the decreased cytotoxicity of the conjugate, greater doses of PP will be required in the tumor environment which could be possible due to the protracted half-life of the carrier albumin.

Conclusions

The utter insolubility of PP requires formulation in a solution containing albumin prior to injection. Several issues arose from this including PP precipitation during formulation, protein aggregation and a decrease in activity with little hydrolysis of the fatty acid. Admittedly, PTX was not an ideal choice for PDG but nevertheless the PP-containing formulation is capable in providing: (1) a solution devoid of any undesirable solvents or

detergents; (2) a cost-effective method for high affinity albumin binding via the simple reaction with PDG's one-step chemistry, (3) similar characteristics to ABRAXANE and (4) superior pharmacokinetics. As is the case with ABRAXANE, albumin may also facilitate increased tumor accumulation which can make PDG a very useful tool in improving cancer drug therapies. This could be true not only in the cancer model, but the success of LEVEMIR demonstrates albumin is a useful carrier for therapies of many disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Synthesis of PDG anhydride. (i) pyridiniumchlorochromate, NaOAc, (carboethoxymethylene)triphenylphosphorane, DCM RT, 85%; (ii) Na⁰, EtOH, diethylmalonate reflux 3 h, 1 eq H₂O, 2 eq NaCl, DMSO reflux 5 h, 94%; (iii) 3 N KOH/ MeOH reflux 3 h, 99%; (iv) trifluoroacetic anhydride, EtOAc, 37°C, 2 h, 100%.

Figure 2. Synthesis of PP. (i) 3-pentadecylglutaric anhydride, DMAP, pyridine, 24 h room temperature, 85%. ^3H corresponds to the sites replaced with tritium in the radiolabeled compounds.

Figure 3. Biochemical stability of PP in undiluted murine serum, CT26 murine tumor cell homogenate and CT26 tumor tissue homogenate. The data are presented as a % of total mass quantified from LC/MS. Error bars represent the standard deviation of matrices performed in duplicate with aliquots quantified in triplicate.

Figure 4. Serum pharmacokinetics for [3H]-PP-HSA and [3H]-TAXOL in CT26 tumor bearing mice. The insert magnifies the terminal phase of each treatment which was used to calculate the half-life, $t_{1/2}$ (Table 2). Error bars represent the standard deviation calculated from $n = 3$ mice with blood samples analyzed in triplicate.

Figure 5. Biodistribution analysis of [^3H]-PP-HSA (**a**) and [^3H]-TAXOL (**b**) in CT-26 tumor bearing mice. Error bars represent the standard deviation calculated from $n = 3$ mice with organ samples analyzed in duplicate.

Table 1

Cytotoxicity of PTX and PP in Various Cancer Cell Lines

TGI		μM	μM
Cancer Type	Cell Line	PTX	PP
Leukemia	HL-60(TB)	0.03	1.78
	RPMI-8226	0.32	2.75
NSCLC	NCI-H522	0.08	2.40
	NCI-H460	0.79	1.35
Colon	HC-2998	0.13	1.95
	KM12	0.16	1.26
CNS	SF-539	0.06	1.78
	SNB-75	0.08	1.20
Melanoma	MDA-MB-435	0.02	0.20
	LOX IMVI	1.00	1.78
Ovarian	OVCAR-3	0.16	0.60
	OVCAR-5	0.79	2.63
Renal	RXF 393	0.50	2.29
	SN12C	1.00	2.69
Prostate	DU-145	0.16	1.23
	PC-3	0.63	3.24
Breast	BT-549	0.79	2.24
	MDA-MB-468	1.00	0.27

Total Growth Inhibition of PP and PTX from the NCI-60 DTP Human Tumor Cell Line Screen. For simplicity, only the TGI data is presented for 2 cell lines representative of each type of cancer. The cell lines were selected as having the highest toxicity for PTX and PP.

Table 2

Dynamic Dialysis of [³H]-PP-HSA complexes

Time (h)	A				B		
	[HSA](μM)	[EtOH]%(v/v)	[PP](μM)	[HSA](μM)	[EtOH]%(v/v)	[PP](μM)	
0	0	10	2.8	0	10	0	
1	0	10	2.1	0	10	0.7	
2	0	10	1.8	0	10	1.0	
3	0	10	1.7	0	10	1.1	
4	0	10	1.5	0	10	1.3	
5	0	10	1.5	0	10	1.3	
0	0	10	2.8	131	10	0	
24	0	10	1.2	131	10	1.6	
0	722	0	467	609	0	0	
24	722	0	455	609	0	12	
48	722	0	454	609	0	14	
0	179	0	127	146	0	0	
24	179	0	123	146	0	4	
48	179	0	115	146	0	4	

[³H]-PP-HSA in compartment **A** was dialyzed against delipidated HSA in compartment **B** across a regenerated cellulose membrane (MWCO 12–14 kDa) to assess the stability of the aggregates. After dialysis the membrane showed minimal PP binding.

Table 3

Pharmacokinetic Parameters of PP-HSA and TAXOL

Treatment	Dose (μg)	C ₀ (μg/mL)	V _d (mL)	t _{1/2} (h)	Cl (mL/h)	AUC _∞ (h·μg/mL)	MRT _∞ (h)
PP	80	12.05	36.56	23.13	1.10	72.59	29.82
TAXOL	150	6.21	116.19	6.61	12.18	12.07	6.53
PP/TAXOL	0.53	1.94	0.31	3.50	0.09	6.01	4.57

Summary of important pharmacokinetic parameters for [³H]-PP-HSA and [³H]-TAXOL. A non-compartmental model was fit to the data using WinNonlin. AUC, area under the curve; C₀, estimated initial serum concentration; Cl, systemic clearance; V_d, volume of distribution; MRT, mean residence time.