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A Comparative *In Vivo* Study of Albumin-Coated Paclitaxel Nanocrystals and Abraxane

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Abstract

Nanoparticulate drug carriers exploit the enhanced permeability of tumor vasculature to achieve selective delivery of chemotherapeutic drugs. For this purpose, nanoparticles (NPs) need to circulate with a long half-life, enter tumors via the permeable vasculature and stay in tumors via favorable interactions with tumor cells. To fulfill these requirements, albumin-coated nanocrystal formulation of paclitaxel (PTX), Cim-F-alb, featuring high drug loading content, physical stability in serum, and surface-bound albumin in its native conformation is prepared. The pharmacokinetic and biodistribution (PK/BD) profiles of Cim-F-alb in a mouse model of B16F10 melanoma show that Cim-F-alb exhibits a longer plasma half-life and a greater PTX deposition in tumors than Abraxane by ~1.5 and ~4.6 fold, respectively. Biolayer interferometry analysis indicates that Cim-F-alb has less interaction with serum proteins than nanocrystals lacking albumin coating, indicating the protective effect of the surface-bound albumin against opsonization in the initial

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

deposition phase. With the advantageous PK/BD profiles, Cim-F-alb shows greater and longer-lasting anti-cancer efficacy than Abraxane at the equivalent dose. This study demonstrates the significance of controlling circulation stability and surface property of NPs in efficient drug delivery to tumors and enhanced anti-cancer efficacy.

Graphical Abstract

A new paclitaxel nanocrystal system (Cim-F-alb) consisting of 90% drug nanocrystal core and 10% albumin bound to the surface outperforms Abraxane at the equivalent dose. Cim-F-alb shows a longer plasma half-life and a higher PTX deposition in tumors with greater and longer-lasting anti-cancer efficacy than Abraxane. Native albumin coating provides protective effect against opsonization in the initial deposition phase.

Keywords

paclitaxel; nanocrystals; Abraxane; albumin; pharmacokinetics

1. Introduction

Nanomedicine has attracted significant interest in cancer therapy because it has potentially selective access to tumors via leaky vascular structure and thus offers an exciting opportunity to improve the safety and efficacy of chemotherapy. The defective, leaky vascular structure has been described as a unique feature of solid tumors in various animal models^[1, 2] and selected cases of human patients.^[3] With new biomarkers of tumor leakiness on the horizon, the enhanced permeability of tumor vasculature remains one of the most important opportunities for selective delivery of chemotherapy.^[4] Accordingly, there is a continuing need for nanomedicine that can effectively leverage the enhanced permeability of tumors.

Drug nanocrystals (NCs) have gained growing interest in the systemic delivery of poorly water-soluble anti-cancer drugs such as paclitaxel (PTX), due to the high drug loading efficiency and circulation stability. The drug loading efficiency of NCs can be > 90 wt%.^[5] NCs with high intermolecular lattice energy remain stable in circulation.^[6] For cancer patients with the leaky peritumoral vasculature, stable NCs are expected to show more favorable pharmacokinetic (PK) and biodistribution (BD) profiles than commercial formulations, such as Taxol (surfactant-solubilized PTX) and Abraxane (albumin-bound PTX), which circulate as smaller particles such as micelles (14 nm)^[7, 8] and albumin-bound drugs (~10 nm),^[9, 10] respectively.

With this expectation, several groups have developed NC formulations of PTX and evaluated their PK/BD profiles.^[11–15] PTX NCs were prepared with an average size ranging from 150

to 250 nm by various methods such as high-pressure homogenization or anti-solvent precipitation.^[12, 14] With no surface protection, these NCs however showed rather unfavorable PK/BD profiles, such as a shorter plasma $t_{1/2}$, less systemic exposure, greater accumulation in the mononuclear phagocytic system (MPS) organs,^[15] and lower tumor accumulation than PTX injectable solutions.^[14] Surface stabilizers such as polymeric surfactants or proteins were used to protect the NCs from opsonin adsorption and MPS recognition and/or to increase target specificity,^[11–13] but the outcomes have been equivocal. PTX NCs stabilized with Pluronic F127 and docusate sodium did not enhance the systemic exposure of PTX but rather increased the liver and spleen accumulation.^[12] PTX NCs with a layer-by-layer polyelectrolyte assembly and polyethylene glycol (PEG) layer on the surface showed higher accumulation in the MPS organs and lower tumor accumulation than Abraxane, due to the premature loss of PEG surface and subsequent opsonization.^[11]

In addition, NC systems reported so far have not shown an improved antitumor efficacy as compared to commercial PTX formulations at the equivalent dose.^[13, 16–18] PTX NCs stabilized with Pluronic polymers were not significantly better than Taxol in delaying tumor growth.^[13, 18] When transferrin (Tf)-coated PTX NCs were used to treat Tf-receptor-expressing KB tumors,^[16] their performance was better than bare NCs (tumor growth suppression rate 45.1% vs. 28.8%) but not superior to Taxol's (tumor growth suppression rate 93.3%). PTX NCs coated with PEG via covalent conjugation improved the stability of surface protection, but their anti-cancer efficacy was not significantly better than those of Abraxane and Taxol.^[17] In most cases, the main advantage of NC systems was limited to eliminating the use of toxic solubilizers rather than improving the therapeutic efficacy of unit dose.

In contrast, albumin-coated NCs that we developed previously showed anti-cancer efficacy superior to Abraxane in C57BL/6 mice bearing B16F10 melanoma, an aggressive cancer model.^[19] The PTX NCs were prepared in two steps – crystallization in medium containing Pluronic F-127 (F127) followed by surface coating with albumin ("Cim-F-alb"). Cim-F-alb showed greater tumor accumulation and anti-cancer efficacy than the same dose of Abraxane. The following features of Cim-F-alb may have contributed to the enhanced anti-cancer effect: (i) an improved stability in circulation, further enhancing drug accumulation in tumors; (ii) albumin surface reducing opsonization^[20, 21] and MPS clearance of the NCs; and (iii) albumin surface facilitating tumor accumulation of the NCs via interactions with albumin-binding proteins such as endothelial gp60^[22] and SPARC (secreted protein acidic and rich in cysteine) proteins located in and near cancer cells.^[23] In the previous study,^[19] we have demonstrated that Cim-F-alb maintains good stability in serum at a blood concentration initially achievable with the intravenous injection. We have also compared cellular uptake of NCs with different albumin conformations in J774A.1 macrophages and SPARC-overexpressing B16F10 melanoma cancer cells and found that Cim-F-alb with native albumin shows favorable cell interaction profiles (relatively low macrophage uptake and good interaction with B16F10 cells).

In this study, we compare PK/BD profiles of Cim-F-alb and Abraxane aiming to examine the impact of the circulation stability and albumin coating on tumor deposition and long-term anti-cancer efficacy of the NCs. The results demonstrate that Cim-F-alb accumulates in

tumors more than Abraxane upon repeated dosing, in part due to the circulation stability and the initial control of protein corona formation. With the advantages in PK/BD profiles, Cim-F-alb extends the median survival of B16F10 tumor-bearing mice to a greater extent than Abraxane at an equivalent dose.

2. Results and Discussion

2.1. NC characterization

Cim-F-alb was a rod-shape nanocrystal with an average length of 260 ± 106 nm and a width of 42 ± 9 nm ($n = 121$) per TEM images (Figure 1). Dynamic light scattering found the z-average of Cim-F-alb to be 190.5 ± 12.9 nm with a polydispersity index (PDI) of 0.215 ± 0.059 and the zeta potential to be -10.1 ± 2.1 mV ($n=6$ independent batches). The average contents of PTX and albumin were 91.0 ± 2.5 wt% ($n=7$) and 11.9 ± 3.2 wt% ($n=3$), respectively. These properties are consistent with our previous report.^[19]

2.2. Pharmacokinetic and biodistribution profiles

We previously observed an improved anti-cancer efficacy of Cim-F-alb compared to Abraxane at PTX dose of 15 mg/kg administered every three days a total of four times ($q3d \times 4$).^[19] Given that both Cim-F-alb and Abraxane have PTX and albumin as their main components (except for a trace amount of F127 contained in Cim-F-alb), we speculated that the structural stability of Cim-F-alb in circulation, supported by its persistent light scattering in serum,^[19] contributed to greater tumor accumulation than Abraxane. To test this hypothesis, we examined the PK/BD profiles of Cim-F-alb and Abraxane.

We first determined the maximum tolerated dose (MTD) for Cim-F-alb with healthy C57BL/6 mice. The reported MTDs of commercial PTX formulations in experimental models vary from 30 to 150 mg/kg as a cumulative dose with some exceptions, depending on the excipients and dosing schedules (Table S1, Supporting Information). Since Cim-F-alb was well tolerated at a dose of PTX 15 mg/kg $q3d \times 4$ times in the previous study,^[19] the dose and frequency were further increased up to PTX 90 mg/kg $q3d \times 5$ times. All mice receiving Cim-F-alb at a dose of PTX 90 or 60 mg/kg each time had to be sacrificed during the treatment period due to the excessive body weight loss (Figure S1, Supporting Information). However, animals receiving Cim-F-alb at a dose of PTX 30 mg/kg $q3d \times 5$ showed neither significant changes in body weight nor outstanding abnormality in blood chemistry (Table S2, Supporting Information). Thus, the MTD of Cim-F-alb was deemed to be PTX 30 mg/kg $q3d \times 5$, comparable to that of Abraxane reported in the literature.^[24]

For comparison of the PK profiles, B16F10 tumor-bearing mice were treated with a single intravenous injection of Cim-F-alb or Abraxane at the dose equivalent to PTX 30 mg/kg (Figure 2). The plasma PTX concentration-time profiles indicated higher PTX concentrations with Cim-F-alb than with Abraxane, especially at later time points (beyond 6 h post-injection). The terminal $t_{1/2}$ in plasma following the dosing of Abraxane was 3.7 h, comparable to the values reported in other studies with mouse models and similar analytical methods.^[11, 25] The $t_{1/2}$ of Cim-F-alb was 5.6 h, ~1.5 times longer than that of Abraxane. The PK parameters obtained via non-compartmental analyses also indicated an enhanced

systemic exposure of PTX with Cim-F-alb. The total area under the plasma concentration-time curve ($AUC_{0-\infty}$) of Cim-F-alb was 1.7 times greater than Abraxane. Accordingly, the total clearance with Cim-F-alb was slower than that with Abraxane. On the other hand, the volume of distribution at steady state (V_{ss}) and mean residence time (MRT) were increased with Cim-F-alb by 1.6 and 2.8 times, respectively, compared to Abraxane. These PK parameters indicate that Cim-F-alb offers an extended circulation time and drug-target residence time, likely due to slower dissolution of crystalline Cim-F-alb than Abraxane, which disintegrates quickly upon intravenous injection.^[10, 24, 25]

In line with the enhanced systemic exposure by Cim-F-alb, the PTX levels of various tissues were also higher with Cim-F-alb than with Abraxane (Figure 2). When tumor tissues were harvested and analyzed at various time points post-injection, the PTX levels were higher with Cim-F-alb than with Abraxane, at all time points compared. The peak PTX levels in tumor tissues were observed at 6 h post-injection: $2.3 \pm 1.2\%$ and $0.5 \pm 0.2\%$ of the injected dose for Cim-F-Alb and Abraxane, respectively. In the liver and spleen, the PTX levels were highest immediately after the injection, but a clear second peak was observed at 6 h post-injection. Although their intensities were relatively small, the second peaks were also apparent in the lung, kidney, and heart in mice that received Cim-F-alb, but not in mice that received Abraxane. The second peaks observed with Cim-F-alb suggest that the major organs receive not only an initial deposition but also a delayed input from the stable Cim-F-alb particles in circulation. With its circulation stability, Cim-F-alb likely afforded advantage over Abraxane in leveraging the enhanced permeability of tumors, thus achieving a high level of PTX in tumors. These results underscore the significance of physical stability in efficient delivery of circulating NPs to tumors. Notwithstanding, other NC formulations with presumably similar stability in circulation did not demonstrate favorable PK/BD profiles compared to commercial PTX formulations.^[11, 12, 14, 15] Therefore, we hypothesized that the surface-bound albumin layer on Cim-F-alb also contributes to its efficient delivery to tumors.

2.3. Serum protein binding to Cim-F-alb

We first examined the impact of albumin coating on the binding of serum proteins to circulating NCs. Adsorption of serum proteins, in particular those tagging foreign subjects for phagocytic removal (opsonization), is the first challenge for the intravenously injected nanoparticles to encounter in circulation.^[26, 27] To examine whether the albumin coating affected the extent and kinetics of opsonization, we prepared PTX NCs with different surface properties (Cim-F-alb with albumin coating, PNC with no protective coating, Cim-C-alb with denatured albumin coating,^[19] or Cim-C with cationic surfactant layer and no albumin), incubated them in undiluted mouse serum, and analyzed the surface-adsorbed proteins (protein corona) by LC-MS and SDS-PAGE. In addition, *in situ* interaction between serum proteins and NCs was monitored by biolayer interferometry (BLI).

2.3.1. Protein corona analysis—Cim-F-alb and PNC were incubated in C57BL/6 mouse serum for 15 min or 3 h. The NCs were washed twice to remove loosely-bound proteins (soft corona) leaving only the proteins adsorbed to NC surface with high affinity (hard corona) and analyzed with SDS-PAGE and LC-MS. We determined that two cycles of

washing were sufficient to remove the soft corona because the protein composition did not further change after the second wash (Figure S2, Supporting Information). After the two cycles of washing, the serum-incubated NCs showed minor or negligible changes in the particle size: the z-average of Cim-F-alb increased from 207.9 ± 15.2 nm to 246.0 ± 17.9 nm after the incubation ($n=3$), and that of PNC from 385.1 ± 28.4 nm to 372.5 ± 29.5 nm ($n=3$). On the other hand, both NCs showed greater negative surface charges indicating the adsorption of serum proteins: the zeta potential of Cim-F-alb changed from -9.4 ± 0.8 mV to -19.9 ± 0.6 mV after the incubation, and that of PNC from -4.0 ± 1.4 mV to -22.6 ± 0.4 mV (Figure S3, Supporting Information).

Proteomic analysis via LC-MS identified α -/ β -globins and lipoproteins such as Apo E, Apo A-IV, and Apo A-I to be mainly bound to the NCs but detected no outstanding differences between Cim-F-alb and PNC (Figure 3). Another NC with denatured albumin coating, Cim-C-alb, also did not show significant difference in protein corona composition. We also used SDS-PAGE band profiles for rough estimation of the extent and composition of protein adsorption. Consistent with LC-MS, no notable difference in corona composition was observed among the three NCs, likely due to inter-experimental variability, which appeared to increase with the incubation time (Figure S4, Supporting Information).

Previous studies reported the formation of distinct protein corona on NPs according to the surface chemistry^[28–30] and identified specific proteins in the corona responsible for various cellular interactions or the lack thereof. Clusterin is reported to be crucial to the stealth effect of polystyrene NPs.^[31] Pre-coating of polystyrene NPs with Apo A-I reduced the uptake by murine macrophages.^[28] Other apolipoprotein proteins (ApoA4 and ApoC3) also reduce non-specific cellular interaction of polystyrene NPs when present on the surface.^[32] On the other hand, Apo-E is involved in endocytosis of NPs by the brain endothelial cells and thus helps deliver NPs to the brain.^[33] Complement component C3 promotes the interaction with macrophage complement receptors and accelerates the removal of NPs.^[34] Since most studies have been performed with inorganic NPs, such as gold,^[30] silica,^[35, 36] or polymeric NPs,^[28, 37] these observations may not be directly applicable to Cim-F-alb, which has a relatively “soft” surface (i.e., non-covalently bound albumin). Moreover, the hard corona analysis is highly dependent on consistent isolation of the surface-bound proteins^[38] and, thus, subject to inter-experimental variability, as we have experienced. This procedure also precludes the identification of soft corona proteins, whose role in nanoparticle function is poorly understood due to the rapid exchange with serum components and the difficulty in separation and identification.^[39–41] To test if the albumin surface affected initial soft corona formation, we resorted to an alternative method to monitor protein binding *in situ*.

2.3.2. Binding kinetics of NCs with serum proteins—The short-term interactions between NCs and mouse serum proteins were evaluated by real-time evaluation of NC binding to serum proteins using BLI. BLI detects molecules adsorbed to a fiber optic biosensor according to the changes in optical interference between reflected beams.^[42, 43] Serum proteins were immobilized to the tip of a biosensor and exposed to NC suspensions for 5 min to monitor their interaction with NCs. The equilibrium dissociation constant (K_D) values were much smaller with PNC than with Cim-F-alb: PNC ($K_{D1} = 5.16$ μ M, $K_{D2} = 0.0079$ μ M) and Cim-F-alb ($K_{D1} = 1300$ μ M, $K_{D2} = 8620$ μ M) (Table S3, Supporting

Information). These results demonstrate a much higher affinity of PNC for serum proteins than that of Cim-F-alb, most likely due to the hydrophobic nature of PNC surface (Figure 4). Cim-C with cationic surface charge and no albumin coating showed even greater binding affinity for serum proteins ($K_{D1} = 0.49 \mu\text{M}$, $K_{D2} = 2.56 \mu\text{M}$) than PNC, indicating electrostatic interactions with negatively charged proteins. This shows that albumin on Cim-F-alb created a hydrophilic, protein-resistant layer, consistent with its known role as a dysopsonin.^[44, 45] It is noteworthy that this difference between Cim-F-alb and PNC was not reflected in the hard corona profiles evaluated after 15 min or 3 h incubation (Figure 3; Figure S4, Supporting Information). It implies that the surface albumin mainly affects the formation of soft corona in the initial circulation phase.

2.3.3. Cell-NC interactions—To investigate if the soft corona takes part in phagocytic removal of NCs, we incubated Cim-F-alb or PNC with J774A.1 murine macrophages in 50% mouse serum (allowing in situ corona formation) or in serum-free culture medium (no protein corona) for 0.5 h and evaluated the NC uptake. The concentration of NCs was equivalent to PTX 30 $\mu\text{g/mL}$, where they remained as crystals for the duration of incubation (Figure S5, Supporting Information).^[19] More PNC was taken up by J774A.1 macrophages in the medium containing 50% serum than those in serum-free medium, suggesting that the corona formed in 0.5 h facilitated their uptake (Figure 5a). On the other hand, macrophage uptake of Cim-F-alb was not affected by 50% serum in 0.5 h. This demonstrates that Cim-F-alb was resistant to the initial corona formation, consistent with the results from BLI analysis.

It is worth noting that the particle uptake profiles were more favorable for Cim-F-alb than PNC. Cim-F-alb and PNC with or without hard corona (formed in 15 min) were incubated with J774A.1 macrophages or B16F10 melanoma cells. Cim-F-alb was taken up less by macrophages and more by B16F10 cells than PNC, irrespective of the corona (Figure 5b). This difference highlights other potential role of albumin coating, such as particle size reduction during the NC formation. As reported previously, albumin coating helps limit crystal growth during production and purification and makes Cim-F-alb smaller than bare NCs^[19] (Cim-F-alb: $190.5 \pm 12.9 \text{ nm}$ by DLS, $n=6$; PNC: $346.8 \pm 27.3 \text{ nm}$ by DLS, $n=4$). In general, smaller sizes are preferred for the uptake by non-phagocytic cells,^[46, 47] and larger particles are more readily taken up by macrophages.^[48] The particle size reduction is a unique feature of albumin coating and may be further optimized.

2.4. Anti-cancer efficacy

Given the favorable PK/BD profiles of Cim-F-alb relative to Abraxane, we wondered whether the anti-cancer efficacy of Cim-F-alb would also be superior to other formulations, especially with more intense dosing regimens (increased doses and frequencies) than our earlier study (15 mg/kg q3d $\times$ 4).^[19] Treatment groups included Cim-F-alb and Abraxane at the pre-determined MTD (30 mg/kg q3d $\times$ 5) (Figure S1, Supporting Information) as well as PNC that was found inferior to Cim-F-alb in the above cell-based studies. None of the surviving animals lost body weight during the treatment (Figure S6, Supporting Information). PNC-treated mice performed poorly compared to those treated with Abraxane and Cim-F-alb. Only 1 out of 6 PNC-treated mice survived by the end of the experiment (13

days after the 1st treatment). Three out of 6 Abraxane-treated mice survived. In contrast, all of the Cim-F-alb-treated animals survived with relatively low tumor growth rates, although not statistically different from Abraxane-treated ones.

Surviving mice were sacrificed one day after the last dose (i.e., 13 days after the 1st treatment) for the evaluation of PTX distribution in major organs (Figure 6). Cim-F-alb showed significantly higher levels of PTX in tumor, liver, and spleen than Abraxane, similar to the observations following a single dose (Figure 2). Upon close comparison, the results from single and multiple dosing revealed additional differences between Cim-F-alb and Abraxane. When PTX accumulation in tumor was compared between Cim-F-alb and Abraxane, the difference was more prominent with multiple dosing: the differences at 24 h post-injection were 5.0 and 1.7 µg/g for multiple and single dosing, respectively. It was not the case for the PTX levels in the liver and spleen at 24 h post-injection. The differences in the liver PTX levels at 24 h post-injection were comparable (7.1 and 9.1 µg/g for multiple and single dosing, respectively). So were the differences in the spleen PTX levels at 24 h post-injection (1.3 and 1.7 µg/g for multiple and single dosing, respectively). This indicates that multiple dosing incurs tumor accumulation of Cim-F-alb to a greater extent than Abraxane, but not in other highly perfused organs such as the liver, spleen, lung, kidney and heart (Figure 6). With a single surviving mouse, comparison between PNC and the other treatments was not feasible, but its tumor level was no higher than those of Cim-F-alb and Abraxane. The tumor growth rate, animal survival, and tumor deposition collectively support that PNC was inferior to Cim-F-alb, as expected from the cell-based studies. Thus, PNC was not included in subsequent studies.

To evaluate if the cumulative Cim-F-alb exerts long-lasting suppression of tumor growth after cessation of the treatment, the same dosing schedule was repeated with another set of mice, monitoring them till the endpoint (Figure 7a). Animals with no drug treatment (PBS group) reached the endpoint with a median survival time of 11 days, reflecting the aggressiveness of the B16F10 tumors.^[49] Abraxane extended the median survival time to 15 days ($p < 0.05$ vs. PBS group by Log-rank test). Cim-F-alb-treated animals showed the longest median survival time (21 days, $p < 0.001$ vs. PBS group). The specific tumor growth rates showed a similar trend, where Cim-F-alb achieved a significant delay in tumor growth ($p < 0.01$ vs. PBS; Tukey's multiple comparisons test). Abraxane appeared to have attenuated tumor growth during the treatment period, but tumors grew soon after the final dose, rendering no significant difference from PBS in the tumor growth rate. This result supports that Cim-F-alb had the anti-cancer efficacy that lasted longer than Abraxane, likely due to the greater accumulation in tumor.

Of note, the tumor concentrations of PTX at 24 h post-injection of the last dose (10.1 µg/g (11.8 µM) for Cim-F-alb and 5.3 µg/g (6.2 µM) for Abraxane) (Figure 6) were far higher than the in vitro IC₅₀ value of PTX (94.3 to 926.7 nM) in B16F10 cells, but tumors continued to grow and all animals succumbed to death. This gap may result from distinct reasons for each treatment. For Abraxane, the drug concentration may not have been maintained sufficiently long in tumors. Cim-F-alb may have advantage over Abraxane in this regard due to the size, but its dissolution takes time. In addition, as NPs Cim-F-alb may not efficiently penetrate into tumors.^[50] This limitation may in part explain why the differential

tumor concentrations of PTX did not translate to statistically significant difference between Abraxane and Cim-F-alb in both median survival time and tumor growth rate. Further efforts need to be made to reduce the NC size so that its dissolution and/or tumor penetration may be enhanced.

In summary, our results with Cim-F-alb demonstrate that a stably circulating nanoparticle form of PTX outperforms the clinical gold standard Abraxane at the equivalent dose, with a minimal involvement of non-drug excipients. Outperformance at the equivalent dose is quite promising given that many new nanoformulations demonstrate superior therapeutic outcomes to commercial benchmarks at respective MTDs, which are higher than those of commercial products.^[24, 51] The low albumin content is also noteworthy. Though effectively controlled by donor screening and product manufacturing processes,^[52] albumin carries a remote risk of viral disease transmission. With the reduced albumin content (12 wt% in Cim-F-alb vs. 90 wt% in Abraxane), Cim-F-alb has a lower risk for disease transmission, if any, than Abraxane.

3. Conclusion

We evaluated PK/BD profiles of albumin-coated NC formulation of PTX, Cim-F-alb, in B16F10 melanoma-bearing C57BL/6 mice. Compared to the clinical benchmark Abraxane, Cim-F-alb showed prolonged circulation and greater accumulation in B16F10 melanoma, by resisting protein adsorption and maintaining NP status during the initial phase of circulation. Consistent with the PK/BD profile, Cim-F-alb showed superior antitumor efficacy that lasted longer than Abraxane at the equivalent dose. These results demonstrate that Cim-F-alb may offer greater therapeutic benefits than existing PTX formulations with a simple change in physical assembly of the components.

4. Experimental Section

Materials

PTX was a gift from Samyang Biopharm (Seoul, Korea). Pluronic F127 (F127) was a gift from BASF (New York, NY, USA). Abraxane was obtained from Celgene Corporation (Summit, NJ, USA). Human serum albumin (>96% agarose gel electrophoresis), *tert*-butyl methyl ether (TBME), and hexadecyltrimethylammonium bromide (CTAB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). C57BL/6 mouse serum was purchased from Innovative Research (Novi, MI, USA). Reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA, USA). All other reagents were purchased from Fisher Scientific (Waltham, MA, USA).

NC production

Cim-F-alb NC was produced by the crystallization in the medium containing Pluronic F-127 and coating with albumin as reported earlier.^[19] Briefly, 6 mg of PTX and 24 mg of F127 were dissolved in 3 mL of chloroform in a round bottom flask, followed by evaporation with a rotary evaporator at 40 °C for 10 min. The resulting film was hydrated with 6 mL of water with bath sonication for 10 s, and the suspension was probe-sonicated for 5 min with an amplitude of 40% and a 1:1 duty cycle every 2 s in ice bath. Then, the suspension was

incubated with 24 mg of human serum albumin for 24 h at room temperature on a rotating shaker. Excess F127 and unadsorbed albumin were removed by centrifugation at 135,700 rcf for 10 min at 4 °C.

Three other types of NC (PNC, Cim-C-alb, and Cim-C) were produced for comparison with Cim-F-alb. First, PNC was produced by anti-solvent and temperature-induced crystallization method.^[53] Briefly, 1 mL of PTX solution in ethanol (4 mg/mL) was added to 20 mL of cold water and stirred for 10 min in a round-bottom flask immersed in a sonication bath. Ethanol was removed by rotary evaporation for 10 min at 40 °C, and the resulting NCs were collected on a polycarbonate membrane filter (pore size 100 nm). PNC was retrieved by sonicating the filter in water. Second, Cim-C-alb was produced in a similar manner as Cim-F-alb except for the replacement of F127 with CTAB. Briefly, 6 mg of PTX and 2.4 mg of CTAB were dissolved in 3 mL of chloroform in a round bottom flask, followed by evaporation with a rotary evaporator at 40 °C for 10 min. The resulting film was hydrated with 6 mL of water with bath sonication for 10 s, and the suspension was probe-sonicated for 5 min with an amplitude of 40% and a 1:1 duty cycle every 2 s in ice bath. Twenty-four milligrams of albumin was added to NC suspension and incubated at room temperature for 24 h on a rocking shaker. The NC suspension was centrifuged at 135,700 rcf for 10 min at 4°C to remove excess surfactant and unadsorbed albumin. Cim-C was produced in the same manner as Cim-C-alb, except for the omission of albumin coating, and collected on a polycarbonate membrane filter (pore size 100 nm).

NC characterization

The particle size and surface charge of NCs, suspended in sodium phosphate buffer (1 mM, pH 7.4) at a concentration of 20–40 µg/mL, were measured by a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). The morphology of NCs was observed with a FEI Tecnai T20 transmission electron microscope (Hillsboro, OR, USA). Samples were mounted on a 300-mesh Cu Grid with Formvar/carbon supporting film and stained with 1% uranyl acetate. The images were analyzed with the ImageJ software (National Institute of Health, Bethesda, MD, USA) for size distribution of NCs.

Protein corona

Cim-F-alb, PNC and Cim-C-alb equivalent to PTX 300 µg/mL were incubated in undiluted mouse serum for 15 min or 3 h at 37 °C with rotation. The NCs were centrifuged at 135,700 rcf for 15 min at 4 °C and washed with PBS two times. The NC pellet (which contained protein corona) was treated with 10 µL of 10 mM dithiothreitol (DTT)/8M urea solution for 1 h at 37 °C to denature proteins and reduce disulfide bonds between cysteines. Cysteines were then alkylated with a 2% solution of iodoacetamide (IAA) in acetonitrile (ACN) at 37 °C for 1 h. Samples were dried by vacuum centrifugation and treated with Lys-C/trypsin (Promega, Madison, WI, USA). Digestion was performed using the Barocycler 2320 (Pressure Biosciences, Inc., Easton, MA, USA) for 1 h at 50 °C (120 cycles of 20 kpsi for 50 s and atmospheric pressure for 10 s). Peptides were purified by MicroSpin C18 columns (Nest Group, Inc., Southborough, MA, USA). Resulting pellets were resuspended in a 3% ACN/0.1% formic acid (FA) solution. The samples were analyzed using the Dionex UltiMate 3000 RSLC Nano System coupled to the Q Exactive™ HF Hybrid Quadruple-

Orbitrap MS (Thermo Scientific, Waltham, MA, USA). The peptides were loaded onto a trap column (20 $\mu\text{m} \times 350\text{ mm}$) and washed with a 98% purified water/2% ACN/0.01% FA at a flow rate of 5 $\mu\text{L}/\text{min}$. The trap column was then switched in-line with the analytical column after 5 min. Peptides were separated by a reverse phase Acclaim PepMap RSLC C18 (75 $\mu\text{m} \times 150\text{ mm}$) analytical column at a flow rate of 300 nL/min. Mobile phase A was 0.01% FA in water, and mobile phase B was 0.01% FA in 80% ACN. The linear gradient started at 5% B and reached 30% B in 80 min, 45% B in 91 min, and 100% B in 93 min. The column was held at 100% B for the next 5 min before being brought back to 5% B and held for 20 min. Samples were injected into the QE HF through the Nanospray FlexTM Ion Source fitted with an emission tip from Thermo Scientific. Data acquisition was performed focusing on the top 20 precursors at 120,000 resolution with an injection time of 100 ms. Database searches of the UniProt_mouse proteins were performed using Mascot Daemon v.2.5.1 (Matrix Science, Boston, MA, USA) with peptide mass tolerance of 0.05 Da and fragment mass tolerance of 0.2 Da. The false discovery rate (FDR) was adjusted to 1%. Among the identified proteins, those with spectral counts fewer than 50 were excluded. The relative abundance of each protein was estimated using the percentage molecular weight normalized spectral count (MWNSC) by the following Equation (1)^[54, 55]:

$$\text{MWNSC}_k = \left(\frac{\left(\frac{SC}{M_w} \right) k}{\sum_{i=1}^n \left(\frac{SC}{M_w} \right) i} \right) \times 100 \quad (1)$$

where SC is the spectral count of protein k and M_w is the molecular weight of the protein k (in kDa).

Alternatively, protein corona was resolved by SDS-PAGE for semi-quantitative analysis. NC pellets, prepared as above, were mixed with 4 volumes of cold acetone and incubated at $-80\text{ }^{\circ}\text{C}$ for 15 min to induce protein precipitation. After centrifugation at 13,000 rcf for 10 min at $4\text{ }^{\circ}\text{C}$, the supernatant was removed, and the protein pellet was redispersed in 4 $\times$ Laemmli sample buffer and heated at $95\text{ }^{\circ}\text{C}$ for 5 min. The samples were resolved in a 12% polyacrylamide gel, stained with QC Colloidal Coomassie Stain, and imaged with Azure C300 (Dublin, CA, USA). The R_f value and the intensity of protein bands were analyzed by the AzureSpot Analysis Software (Dublin, CA, USA). The R_f value was used to estimate the molecular weight of protein band against standard markers, and the intensity of each protein band was divided by the sum of total protein bands to determine % total protein.

Biolayer interferometry (BLI)

The binding kinetics of NCs to mouse serum proteins were evaluated by biolayer interferometry using an Octet Red384TM system (Pall ForteBio, Fremont, CA, USA). The assays were performed in a 96 well plate with 200 μL of ligands (mouse serum proteins) and analytes (NC suspension) in each well. Mouse serum (50 $\mu\text{g}/\text{mL}$) was loaded on an amine reactive second generation (AR2G) biosensor via EDC/NHS coupling. Different concentrations of PNC, Cim-F-alb, and Cim-C (PTX equivalent 3.125 to 200 $\mu\text{g}/\text{mL}$) in 10 mM phosphate buffer (pH 7.4) were allowed to associate with the biosensor for 300 s, followed by dissociation for 600 s. Non-specific binding of NCs to the sensor was subtracted

from the signal. The binding data was fitted to a global fit 2:1 heterogeneous ligand model using the Octet Data Analysis software to determine the K_D , k_{on} , k_{dis} , X^2 , and R^2 .

Cellular uptake of NCs

J774A.1 murine macrophage cells (ATCC, Manassas, VA, USA) were grown in DMEM medium containing 10% fetal bovine serum (FBS) and penicillin (100 IU/mL) and streptomycin (100 µg/mL). B16F10 mouse melanoma cells (ATCC, Manassas, VA, USA) were grown in RPMI-1640 medium containing 10% FBS, penicillin (100 IU/mL), and streptomycin (100 µg/mL). Cells were seeded in a 6 well plate at a density of 100,000 cells per well. Following overnight incubation, the cell culture medium was replaced with the medium containing Cim-F-alb or PNC. Here, the NCs were suspended in serum-free medium or medium supplemented with 50% mouse serum (MS) to provide the final concentration of PTX 30 µg/mL. Alternatively, the cells were incubated with NCs containing pre-formed corona. The NCs were incubated in undiluted MS for 15 min in 37 °C at the final PTX concentration of 300 µg/mL and washed with PBS two times by centrifugation at 135,700 rcf for 10 min at 4 °C. The corona-coated NCs were provided to the cells as suspended in serum-free medium at the concentration equivalent to 30 µg/mL PTX.

After incubation with each treatment for specified times, cells were washed with PBS two times to remove unbound NC. The cells in each well were collected and suspended in 500 µL of medium. Twenty microliters of the cell suspension was analyzed with a flow cytometer (Accuri C6, BD Sciences, San Jose, CA, USA) to determine the cell number. The rest of cell suspension was analyzed for the intracellular PTX content. The cell pellet was lysed with 3 cycles of freeze-thaw and probe sonication. PTX in the cell lysate was extracted by TBME with carbamazepine as an internal standard and analyzed with HPLC using an Agilent 1100 HPLC system (Palo Alto, CA, USA) equipped with Ascentis C18 column (250 mm × 4.6 mm, particle size 5 µm). The mobile phase was 50% ACN and run at a flow rate of 1 mL/min. PTX was detected by a UV detector at a wavelength of 227 nm.

Maximum tolerated dose

All animal procedures were approved by Purdue Animal Care and Use Committee, in conformity with the NIH guidelines for the care and use of laboratory animals. The maximum tolerated dose (MTD) of Cim-F-alb was determined using 7-week-old healthy male C57BL/6 mice (Envigo; Indianapolis, IN, USA). The animals were acclimatized for 1 week and administered with different doses of Cim-F-alb (15, 30, 60, and 90 mg/kg PTX) via tail vein injection every 3 days for 5 times ($n=3$ per dose; except for 15 mg/kg, $n=1$). The mice were monitored daily over a period of 2 weeks after the first injection. Blood was collected in clot-activating silica coated tubes by cardiac puncture and submitted to the Clinical Pathology Laboratory at Purdue University for blood chemistry analysis. The highest dose tolerated without >20% of initial body or other signs of toxicity was considered the MTD of the treatment. Animals showing >20% weight loss or abnormal signs were humanely sacrificed.

Pharmacokinetics and biodistribution

Male C57BL/6 mice (6–8 weeks) were inoculated with B16F10 melanoma cells (10^6 cells/animal) via subcutaneous injection in the upper flank of the right hind leg. When the average tumor size reached 100 mm^3 (9–10 days post inoculation), mice received a single dose of Abraxane or Cim-F-alb equivalent to PTX 30 mg/kg by tail vein injection. At pre-determined time points (0.5, 1, 3, 6, 12, and 24 h post-injection), 3 mice were randomly selected and sacrificed for blood sampling and collection of major organs. The mice were anesthetized with isoflurane, and blood was collected in heparin coated tubes by cardiac puncture. Plasma was collected by centrifugation at 1000 rcf for 5 min and kept frozen at -80°C . Major organs (heart, lung, liver, spleen, and kidney) were also harvested and stored at -80°C until analysis.

Anti-cancer activity

B16F10 melanoma-bearing mice were prepared in the same manner as above. When the average tumor size reached 100 mm^3 , mice received a total of 5 times of Abraxane ($n=6$), Cim-F-alb ($n=6$), or PNC ($n=6$) (equivalent to 30 mg/kg PTX) every 3 days via tail vein injection. One day after the last dose (13 days after the 1st treatment), surviving mice were sacrificed, and tumor and major organs were excised and stored at -80°C until analysis. Another set of animals were treated with PBS ($n=7$), Abraxane ($n=5$), or Cim-F-alb ($n=7$) (equivalent to 30 mg/kg PTX) in the same dosing schedule except that they were monitored until the endpoint was reached.

During the survival period, tumor volume and body weight were monitored every day. The length (L) and width (W) of each tumor were measured with a digital caliper, and the volume (V) was calculated per the modified ellipsoid formula: $V = (L \times W^2)/2$.^[56] Specific growth rate of a tumor was calculated as $\log V/t$ (V : volume in mm^3 , t : time in days).^[57] Weight loss in excess of 20% body weight or tumors reaching >10% body weight were considered the endpoint.

LC-MS analysis of PTX in tissues and plasma

Tumor and major organs were weighed and homogenized in PBS (0.1 g tissue per mL) with an Omni Tissue Master 125 homogenizer (Kennesaw, GA, USA). For 100 μL of each tissue homogenate and plasma, 40 ng of docetaxel was added as an internal standard and briefly mixed with 1 mL of TBME by vortex mixing. After centrifugation at 3724 rcf for 10 min, 900 μL of TBME phase was separated, evaporated, and reconstituted in 100 μL of a 1:1 mixture of ACN and 0.1% FA in water. An individual standard curve was prepared by spiking PTX in respective matrices (respective tissue homogenates or plasma) to yield the final concentrations of 10, 50, 100, 500, 1,000, 10,000, and 100,000 ng/mL and processed in the same way as the samples. PTX quantification was performed with a 6550 iFunnel Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Three microliter of sample was injected to a Zorbax Extend C_{18} column (2.1 mm $\times$ 50 mm, particle size 1.8 μm) kept at 35°C (Agilent Technologies, Santa Clara, CA, USA). Mobile phase A was 0.1% FA in water, and mobile phase B was methanol. It was run isocratically at 40% A/60%B with a flow rate of 0.4 mL/min. The column was held at 90% B for 5 min between injections. Mass spectral data were obtained in positive ion mode with a Dual Agilent Jet Stream, where

drying gas temperature was 200 °C, and sheath gas temperature was 350 °C with a flow rate of 11 L/min. Scan spectra were acquired over the m/z range of 700–1000. As parent ions, [PTX+Na]⁺ and [DTX+Na]⁺ were monitored at the m/z ratios of 876.3 and 830.3, respectively. Peak ratios of PTX to DTX were plotted against nominal concentration of PTX to generate a calibration curve from each tissue.

The plasma PTX concentration-time profiles were obtained by pooling the data from multiple mice (a single point per mouse, 3 mice per sampling time). To estimate the PK parameters, non-compartmental analyses were performed using the sparse data option of WinNonlin (version 7.0, Certara, NJ, USA), allowing computation of the standard errors (SE) associated with estimated parameters.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (La Jolla, CA, USA). All data were analyzed with one- or two-way ANOVA test followed by recommended multiple comparisons test. A p -value less than 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Transmission electron microscopic image and size distribution of Cim-F-alb
Size distribution was obtained by ImageJ analysis of TEM images (number of the analyzed Cim-F-alb = 121)

Figure 2. Pharmacokinetics and biodistribution of Cim-F-alb and Abraxane after single dose B16F10 tumor bearing C57BL/6 mice received a single dose (equivalent to PTX 30 mg/kg) of Abraxane (blue; $n=3$ per each time point) or Cim-F-alb (red; $n=3$ per each time point) by tail vein injection. PTX content in plasma and tissues was determined by LC-MS. PK parameters were obtained from the non-compartmental analyses.

Figure 3. Protein corona on PNC, Cim-C-alb, and Cim-F-alb measured by LC-MS/MS proteomics analysis

PNC, Cim-C-alb, and Cim-F-alb were incubated in mouse serum for 15 min or 3 h. % total adsorbed protein was calculated using the percentage molecular weight normalized spectral count (MWNSC).

Figure 4. Binding of PNC, Cim-C, and Cim-F-alb to mouse serum proteins immobilized to a fiber optic biosensor, determined by biolayer interferometry (BLI)

(a) PNC, (b) Cim-C, and (c) Cim-F-alb. Black: raw sensorgram data; red: curve fit of sensorgram by a 2:1 heterogeneous ligand model. Dotted vertical line separates the association and dissociation phase. Each curve indicates different NC concentrations: 235 μM to 15 μM (top to bottom) for PNC and Cim-F-alb. 23.5 μM to 367 nM (top to bottom) for Cim-C.

Figure 5. NC-cell interactions

(a) Intracellular PTX content in J774A.1 cells treated with PNC or Cim-F-alb in medium containing 50% mouse serum (MS) or serum-free medium for 0.5 h ($n=3$). *: $p < 0.05$; ****: $p < 0.0001$ by two-way ANOVA with post-hoc Sidak's multiple comparisons test. (b) Intracellular PTX content in J774A.1 cells treated with NCs $\pm$ 15 min-corona (corona formed for 15 min) for 30 min in serum-free DMEM medium ($n=6$) and B16F10 cells treated with NCs $\pm$ 15 min-corona for 3 h in serum-free RPMI-1640 medium ($n=3$). *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$ by two-way ANOVA with post-hoc Sidak's multiple comparisons test.

Figure 6. Biodistribution of Abraxane, Cim-F-alb, and PNC at PTX 30 mg/kg q3d $\times$ 5 (at 24 h post last injection)

Abraxane (blue; $n=3$), Cim-F-alb (red; $n=6$), or PNC (green; $n=1$). PTX content in each tissue was determined by LC-MS. *: $p < 0.05$ by two tailed t-test with post-hoc Mann-Whitney test between Abraxane and Cim-F-alb.

Figure 7. Anti-cancer activity of Abraxane and Cim-F-alb at PTX 30 mg/kg q3d × 5 PBS (black; $n=7$), Abraxane (blue; $n=5$), or Cim-F-alb (red; $n=7$). (a) Dosing schedule. (b) Changes of individual B16F10 tumor volume. Arrows indicate the times of treatment. (c) Body weight change of treated mice. (d) Kaplan–Meier survival curve. *: $p<0.05$; ***: $p<0.001$ vs. PBS by Log-rank test. (e) Specific growth rate of B16F10 tumor. $\log V/t$ (V : tumor volumes; t : time in days). **: $p<0.01$ by Dunn's multiple comparisons test.