Solid lipid nanoparticles of paclitaxel strengthened by hydroxypropyl-β-cyclodextrin as an oral delivery system

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Abstract. The objective of this study was to evaluate the potential of surface-modified paclitaxel (PTX)-incorporated solid lipid nanoparticles with hydroxypropyl-β-cyclodextrin (smPSH). The smPSH released 89.70±3.99% of its entrapped PTX within 24 h when placed in dissolution medium containing sodium lauryl sulfate. The cellular uptake of PTX from smPSH in Caco-2 cells was 5.3-fold increased compared to a PTX solution based on a Taxol formulation. Moreover, smPSH showed an increased cytotoxicity compared to PTX solution. In addition, AUC (5.43 μg•h/ml) and C_{max} (1.44 μg/ml) of smPSH were higher than those (1.81 μg•h/ml and 0.73 μg/ml) of PTX solution. The drug concentration of smPSH (11.12±4.45 ng/mg of lymph tissue) in lymph nodes was higher than that of the PTX solution (0.89±0.75 ng/mg of lymph tissue), suggesting that more PTX was transported to the lymphatic vessels in the form of smPSH. In conclusion, smPSH have a potential as an alternative delivery system for oral administration of PTX.

Introduction

Paclitaxel (PTX) has demonstrated an effective chemotherapeutic activity against a variety of tumors such as drug-resistant ovarian cancer, metastatic breast cancer, non-small cell lung cancer and AIDS-related Kaposi's sarcoma (1). However, the oral bioavailability of PTX is mainly limited by cytochrome P450 activity and drug transporters such as p-glycoprotein (p-gp) in the gut wall and liver (2). PTX is practically insoluble in water (<0.5 mg/l). It is a class IV drug under the biopharmaceutical classification system (BCS) with poor solubility and absorption. The use of Cremophor EL in the commercial formulation (Taxol) of PTX is associated with hypersensitivity reactions such as neurotoxicity and nephrotoxicity in addition to extraction of plasticizers from intravenous infusion lines. Thus, a great deal of effort has been focused on the development of Cremophor EL-free alternative carrier systems including liposomes (3), cyclodextrin (4), emulsions (5), mixed micelles (6), microspheres (7) and polymeric nanoparticles (8). Recently, Abraxane, which is an albumin-bound form and the first solvent-free nanoparticle of PTX with a mean particle size of approximately 130 nm was commercialized (9-12). However, these systems involve injections, thus oral alternatives to intravenous PTX administration could be advantageous.

Solid lipid nanoparticles (SLNs) are well tolerated in living systems since they are constructed from physiological compounds and, therefore, are easily metabolized. Consequently, they play an important role as drug delivery systems for intravenous, peroral, parenteral, pulmonary or ocular administration, for topical delivery (13,14). In addition, distinct advantages of SLNs include the solid state of the particle matrix, the ability to protect labile ingredients against chemical decomposition, and the possibility to modulate drug release (15).

Hydroxypropyl-β-cyclodextrin (HPCD) is more water-soluble than the parent molecule, β-cyclodextrin, and has hydroxypropylester groups attached to the hydroxyl groups in position 2 (16). In addition, HPCD is known to form inclusion complexes with many compounds, which prevents the oxidation of oils and involatile flavors, and solubilizes insoluble compounds (17).

Therefore, the aim of this study was to modify SLNs of PTX (PS) into smPSH using HPCD to achieve higher solubility and cellular uptake of PTX and to evaluate the potential of smPSH.

Materials and methods

Materials. PTX was a gift sample received from SamyangGenex (Daejeon, Korea). Stearic acid was purchased from Daejung Chemical (Cheongwon, Korea). Poloxamer 188 was obtained from BASF (Ludwigshafen, Germany) and lecithin was purchased from Junsei Chemical Co. (Tokyo, Japan). HPCD and mannitol were purchased from Sigma (Steinheim,

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Switzerland). All the solvents were analytical grade and used without further purification. Acetonitrile was obtained as high-performance liquid chromatography (HPLC) grade from JT Baker, Inc. (Phillipsburg, NJ, USA).

Cell cultures. Caco-2 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Caco-2 cells (passage no. 46-52) were cultured in DMEM supplemented with 10% FBS, 1% NEAA, 100 U/ml penicillin and 0.1 mg/ml streptomycin in an incubator with a 5% CO_2 atmosphere and 95% humidity at 37˚C.

Preparation of smPSH. The smPSH were manufactured using a modified hot sonication method. Briefly, 100 mg of stearic acid was melted at 80˚C in a water bath. Five milligrams of PTX was dissolved in 0.25 ml of ethanol and then injected into molten stearic acid under sonication. Lecithin (75 mg) and poloxamer 188 (75 mg) were dispensed into 3 ml of distilled water, sonicated for 10 min at 80˚C in a water bath using a probe type and then added into molten stearic acid and PTX solution under sonication under the same condition. Subsequently, HPCD was added to the above dispersion and incubated for 40 min. The final samples were freeze-dried until further use and named smPSH. The dispersion prior to adding HPCD was freeze-dried using mannitol and termed PS. As a control, PTX solution based on the Taxol formulation was prepared with ethanol containing Cremophor EL.

Analysis of yield, drug loading and encapsulation efficiency of smPSH. The smPSH according to the amount (80.5 mg) of PTX were solubilized with 10 ml of ethanol, heated at 70˚C for 30 min and then cooled down at -20˚C for 30 min. This solution was centrifuged at 3,000 rpm for 5 min to precipitate the undissolved solid stearic acid, filtered through a 0.2-μm filter and injected into the HPLC system. Yield, drug loading and encapsulation efficiency were calculated as follows (18).

Yield (%) = weight of particles/weight of the feeding polymer and drug x100.

Drug loading (%) = weight of the drug in particles/weight of the particles x100.

Encapsulation efficiency (%) = weight of the drug in particles/weight of the feeding drugs x100.

Determination of the physicochemical properties of smPSH. The particle size and ζ-potential analyses of smPSH or PS were performed using laser scattering analyzer (ELS-8000; Otasuka Electronics, Japan). The smPSH or PS was added to the sample dispersion unit and sonicated in order to minimize the inter-particle interactions. The obscuration range was maintained between 20 and 50%. The instrument was set to measure the sample 50 times, and the average volume mean diameter was obtained. The morphology and surface characteristics of the particles were examined by scanning electron microscopy (SEM) (JEOL JSM7500; Thermo Scientific, USA). The samples were mounted onto metal stubs using double-sided adhesive tape onto which the samples were applied. The stubs were sputter-coated with gold particles in a sputter coater for 2 min. The smPSH or PS was characterized using FT-IR (Nicolet 380 FT-IR; Thermo) in order to assess changes in the solid state of the samples. Accurately weighed smPSH or PS of approximately 5 mg was analyzed in aluminium pans on a differential scanning calorimeter (DSC S-650; Scinco, Korea). The DSC runs were conducted from 20 to 150˚C at a rate of 10˚C/min. The specific surface area of smPSH or PS was measured using a BET method.

HPLC systems. An Agilent 1100 liquid chromatography system with an autosampler and UV detector were used. The column used was a C_{18} column (4.0x250 mm, 5-μm particle size, Supelco™; MetaChem, USA). The flow rate of the mobile phase was 1 ml/min and the detection wavelength was set to 227 nm. The mobile phase was a mixture of water and acetonitrile (55:45 v/v). All procedures were carried out at ambient temperature.

Solubility. Accurately weighed smPSH or PS was added to microcentrifuge tubes containing 1 ml of distilled water. The samples were put on an end-to-end labquake rotator (Barnstead Thermolyne, Sparks, NV, USA) at 8 rpm at ambient temperature for 72 h in order to achieve equilibrium and were then stored at room temperature to investigate the change in solubility according to elapsed time. The samples were filtered with a 0.45-μm membrane filter (Dismic-25; Whatman Ltd., Japan) and the absorbance of the filtrate was measured using HPLC. All solubility determinations were performed in triplicate.

In vitro release study. *In vitro* release of smPSH or PS was evaluated using a dialysis bag [molecular weight cut-off of 7000 (Membra-Cel; Viskase, Inc., Chicago, IL, USA)], which was filled with an amount according to 20 μg of PTX and immersed in 30 ml of distilled water including 1% of sodium lauryl sulfate. Aliquots of 3-ml samples were withdrawn from the medium and replaced with the same volume of fresh dissolution medium at an indicated time. The withdrawn samples were estimated using the HPLC system.

Cytotoxicity assay. After a 72-h incubation of the cells (70% confluent) with smPSH or PS, the cytotoxicity was determined by MTT assay according to the manufacturer's protocol (Sigma, USA). Briefly, after incubation, MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma)] was added to each well and incubated for 2 h at 37˚C. The crystals of viable cells were solubilized in isopropanol. The absorbance was determined at 570 nm using a microplate reader (Sunrise; Tecan, Austria). Cell viability (%) was represented as: OD of sample-treated cells/OD of cells incubated without samples) x 100.

Cellular uptake study. One day before the uptake experiments, Caco-2 cells in 6-well plates were seeded at a density of $5x10^5$ cells/well. The cells were washed twice with serum-free DMEM, and then exposed to either 5 μM of the PTX solution, smPSH or PS for 24 h. Subsequently, the cellular uptake studies were terminated by aspirating the media and washing the cells three times with phosphate-buffered saline (PBS). The cells were then lysed with 400 μl of 0.2 N NaOH and sonicated for 10 min. Aliquots (300 and 100 μl) of the cell lysate solution were removed for analysis of PTX and protein content, respectively. The cells were then extracted with 5 ml of ether.

After vortexing for 10 min, the mixture was centrifuged at 4,000 rpm for 15 min. The organic phase was transferred to the other tubes and evaporated to dryness using a vacuum system. The residues were dissolved in 100 μl of MeOH and injected into the HPLC system for analysis. The amount of protein in each sample was determined by the BCA method (Sigma).

In vivo studies after oral administration. All animal studies were conducted in accordance with the 'Guiding Principles in the Use of Animals in Toxicology' adopted by the Society of Toxicology (USA), and the experimental protocols were approved by the Animal Care Committee of Chungnam National University. Male Sprague-Dawley male rats weighing 240-260 g were purchased from the Dae Han Laboratory Animal Research Co. (Chungbuk, Korea). The rats were given commercial rat chow diet (no. 322-7-1) purchased from the Superfeed Co. (Gangwon, Korea) and tap water *ad libitum*. The animals were housed 3/cage in laminar flow cages that were maintained at 22±2˚C, and 50-60% relative humidity. The animals were kept in these facilities for at least one week prior to the experiment and were fasted for at least 24 h before commencement of the experiments. The animals were divided into 3 groups. PTX solution based on a Taxol formulation, smPSH or PS was administered by oral gavage. The oral dose was 25 mg/kg as PTX amount. The blood samples (0.8 ml) were withdrawn from the retroorbital plexus using a disposable capillary tube that was pre-rinsed with heparin sodium in normal saline (20 IU), placed into microcentrifuge tubes at 0, 0.5, 1, 2, 4, 8 and 12 h after drug administration and centrifuged (13,000 rpm, 10 min). Thus, plasma was collected and stored at -70˚C until further analysis. Simultaneously, after 12 h, rats were sacrificed and the lymph nodes were collected. For the determination of PTX in the lymph nodes, 0.3 ml of lymph fluids was placed in the microcentrifuge tubes, the same volume of ethyl ether was added and vortexed for 10 min. The organic layer was separated and evaporated with nitrogen. The residue was reconstituted with 100 μl of mobile phase, analyzed by HPLC and the amount of PTX per lymph tissue amount was determined.

Pharmacokinetic analysis. Non-compartmental pharmacokinetic analysis was carried out using the BE computer program, which calculates the AUC (area under the curve) of the plasma concentration (C_p) as a function of time (t). The maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) were obtained from the experimental data. The area under the plasma concentration curve as a function of time (AUC_{0-t}) from time zero to the time of the last measured concentration (C_{last}) was calculated using the linear trapezoidal rule. The AUC zero to infinite ($AUC_{0-\infty}$) was obtained by adding the AUC_{0-t}, and the extrapolated area was determined by C_{last}/K_{el}. The total plasma clearance (CL/F) was calculated by dividing the dose by the AUC.

Statistical analysis. A Student's t-test or one way-ANOVA was used to compare the groups. A P-value <0.05 was considered to indicate a statistically significant result. All data are expressed as the means ± standard deviation from three independent experiments.

Results

Physicochemical characteristics of smPSH. The morphologic image of PTX showed micrometer-sized planar particles. The particle size of smPSH was 251.40±12.0 nm and that of PS was 302.10±11.69 nm, respectively. On the other hand, the BET surface area of smPSH was 4.79 m^2/g and that of PS was 4.09 m^2/g, respectively. In addition, smPSH showed a high encapsulation efficiency of 71.02±0.70% compared to PS (66.20±0.14%). Thus, additional studies such as a solubility test were carried out to determine the consequences of this phenomenon. The aqueous solubility (0.3 μg/ml) of PTX was saturated to 4.48±0.09 or 4.98±0.18 μg/ml by PS or smPSH, respectively. Importantly, according to the elapsed time after saturation, the solubility of PTX in PS was gradually decreased from 3.64±0.04 to 3.08±0.06 μg/ml in 3-7 days and that of PTX in smPSH was maintained at 4.94±0.07 μg/ml for 3 days. To confirm the stability, ζ-potential was examined. The ζ-potential value of smPSH was -51.37±5.46 mV, which was smaller than that of PS (-37.97±2.51 mV) (Fig. 1A). In contrast, the FT-IR spectra of smPSH are shown in Fig. 1B. The major peaks of PTX were approximately 1250 cm^{-1} (c-o-c stretching). The major peaks of lipid were approximately 2850 and 1670 cm^{-1}. FT-IR spectra for PS and smPSH had two major peaks of lipid.

In vitro release studies. Fig. 2 shows the cumulative release of PTX from PTX solution based on a Taxol formulation, PS or smPSH, respectively. PTX solution showed a prompt release of 90% of PTX within 1 h. The smPSH exhibited a controlled release of 89.70±3.99% of PTX up to 24 h with 36.54±4.81% of a burst release in 1 h. PS exhibited a controlled release of 92.90±3.55% of PTX up to 24 h with 25.56±3.61% of a burst release in 1 h.

Cytotoxicity study. Cytotoxicity was carried out to examine the toxicity of smPSH against Caco-2 cells which showed high dissolution. The smPSH exhibited 52.7±1.82% cell viability while PS demonstrated 64.0±5.47% at the concentration of 5 μM PTX. As a reference, PTX solution exhibited 71.45% cell viability (data not shown) (Fig. 3).

Cellular uptake. Based on the cytotoxicity results, the cellular uptake of PTX solution, PS or smPSH was measured maintaining a concentration of 5 μM PTX. When treated with PTX, the cellular uptake showed a value under LOQ, not absorbing into Caco-2 cells. However, PS showed a cellular uptake of 0.30±0.13 $\mu g/\mu g$ and smPSH showed an uptake of 1.12±0.21 $\mu g/\mu g$. On the other hand, the cellular uptake of PTX solution was 0.21±0.1 $\mu g/\mu g$ (Fig. 4).

In vivo studies after oral administration. The biopharmaceutical aspects of oral absorption of PTX from smPSH were studied in rats. Fig. 5A shows the plasma-time concentration curve for PTX after oral administration of 25 mg/kg of PTX in the form of smPSH, PS or PTX solution. The pharmacokinetic parameters of PTX solution, PS or smPSH after oral administration are shown in Table I. The AUC for smPSH, PS or PTX solution was 5.43±0.51, 4.54±0.72 or 1.81±0.24 $\mu g \cdot h/ml$, respectively. The C_{max} for smPSH, PS or PTX solution was 1.44±0.28, 1.29±0.35 or 0.73±0.14 μg/ml, respectively. The

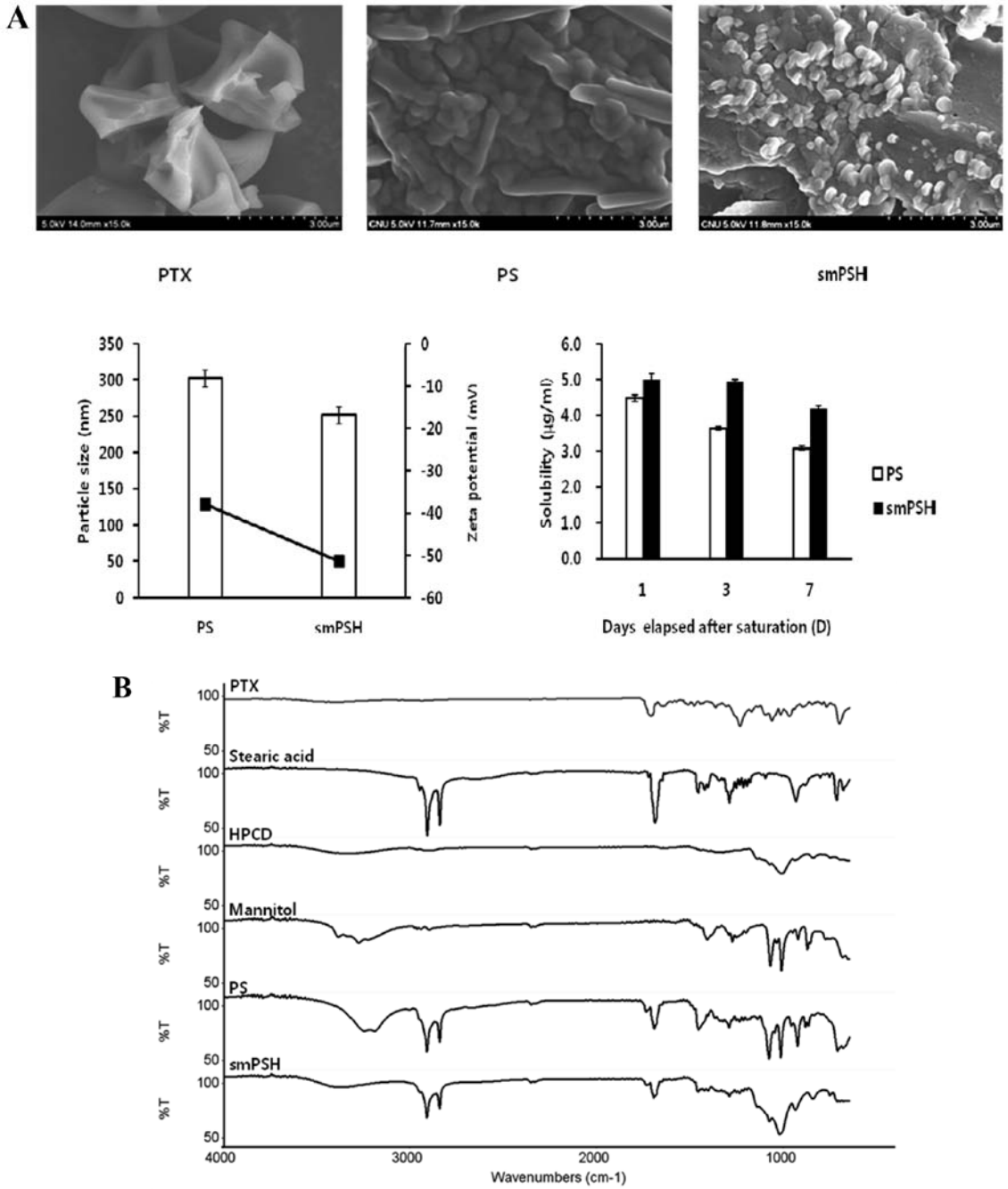

Figure 1. Physicochemical characteristics of smPSH compared to PS. (A) Scanning electon microscopy (SEM), particle size, surface area, solubility, ζ-potential and encapsulation efficiency, successively; (B) FT-IR spectrum.

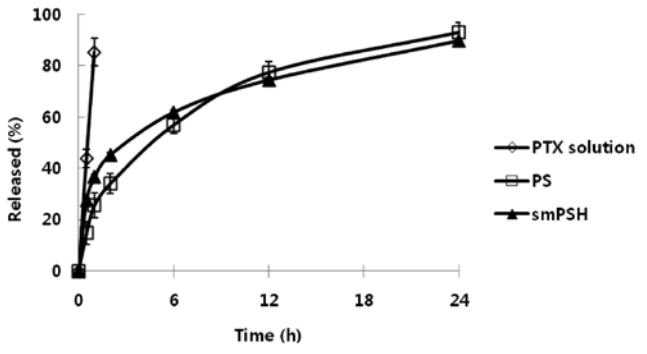

Figure 2. *In vitro* release profile of PTX from PTX solution, PS or smPSH in distilled water including 1% of sodium lauryl sulfate (n=3).

PTX amounts of the different formulations of oral administration (PTX solution, PS or smPSH) noted in the lymph nodes of the rats were 0.89±0.75, 9.05±1.54 or 11.12±4.45 ng/mg of lymph tissue, respectively (Fig. 5B).

Discussion

For improving solubility and increasing cellular uptake of PTX into Caco-2 cells, PTX-incorporated SLN (PS) and surface-modified PTX-incorporated solid lipid nanoparticles with hydroxypropyl-β-cyclodextrin (smPSH) were prepared and evaluated by physicochemical and biopharmaceutical characterization.

Table I. Pharmacokinetic parameters (mean ± SD, n=3) of the PTX solution, PS and smPSH after oral administration.

Parameter	PTX solution	PS	smPSH
C_{max} (μg/ml)	0.73±0.14	1.29±0.35	1.44±0.28
T_{max} (h)	1.00±0.00	1.00±0.00	1.00±0.00
AUC (μg·h/ml)	1.81±0.24	4.54±0.72	5.43±0.51
MRT (h)	2.33±0.19	3.26±0.41	4.05±0.45
$t_{1/2}$ (h)	1.56±0.11	2.03±0.31	2.65±0.38

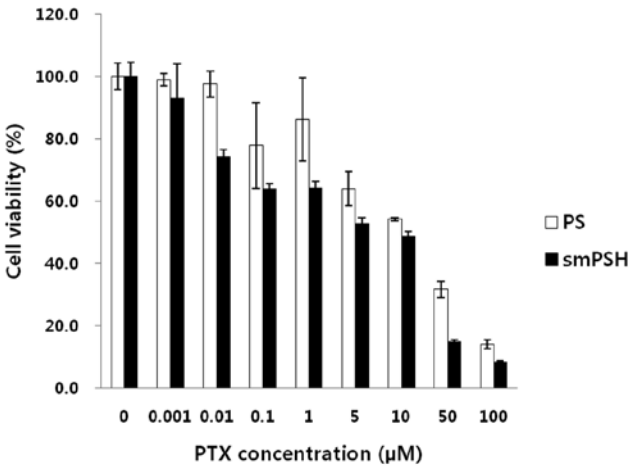

Figure 3. Cytotoxicity of the PTX solution from PS or smPSH (n=3).

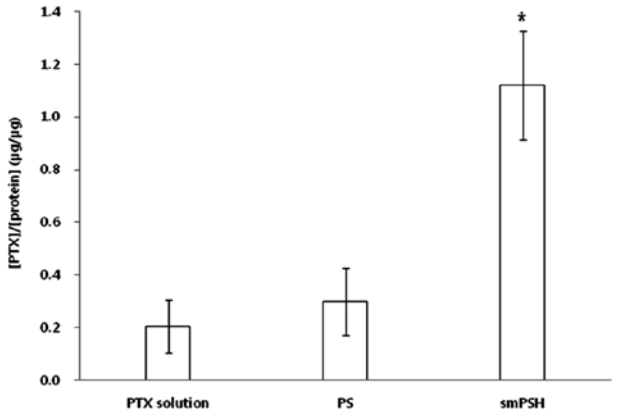

Figure 4. Cellular uptake of PTX from PTX solution, PS or smPSH with 5 μM PTX following incubation at 37˚C for 24 h (n=3). *P<0.05 compared to PTX solution.

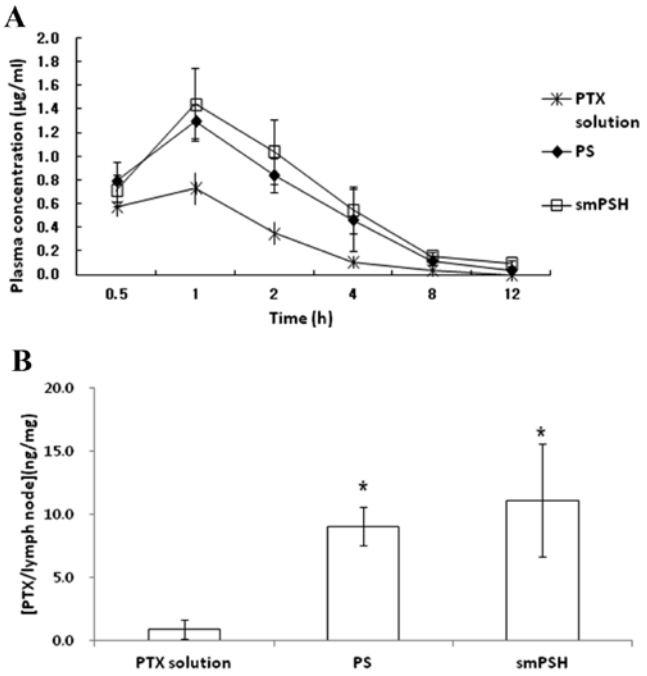

Figure 5. (A) Plasma concentration-time profiles of PTX after oral administration with 25 mg/kg as PTX and (B) the amount of PTX amount in lymph nodes from the PTX solution, PS or smPSH (B) (n=3). *P<0.05 compared to PTX solution.

First of all, the physicochemical properties of smPSH and PS were characterized. The particle size of both smPSH and PS was decreased compared to micrometer-sized PTX. Notably, smPSH had smaller and more spherical particles than PS. The particle size of smPSH was 0.83-fold smaller compared to PS, and the BET surface area of smPSH was 1.2-fold increased compared to PS. It was observed that the solubility of PS or smPSH was increased 15- or 17-fold compared to PTX through additional studies such as solubility test. This may be derived from the reduction in particle size and the increase in the surface area of the particles. Notably, according to the elapsed time after saturation, the smPSH were a more stable formulation having a high solubility of PTX. The ζ-potential value of smPSH is smaller than that of PS and in general, particles are dispersed stably when the absolute value of ζ-potential is above 30 mV due to the electric repulsion between particles (19). On the other hand, the major peaks of PTX were absent in the FT-IR spectra for smPSH indicating that a chemical interaction occurred between lipid and HPCD which is most probably indicative of the surface modification of lipid incorporating PTX by HPCD by FT-IR spectra.

Based on the increase in the solubility and surface area of smPSH compared to that of PS, the increase in the dissolution of smPSH was expected. The burst release may be the rapid release of a drug into the blood or organs causing the concentration to quickly rise then briefly plateau. This may cause an excess of the drug concentration in the therapeutic range which is usually initially observed, follow by the slow and incomplete release of the drug (19). In this study, regardless of the initial burst release, almost complete release of PTX in PS or smPSH was achieved.

The smPSH showed 1.2-fold higher toxicity compared to PS. This was due to the increase in solubility of PTX in smPSH. The cellular uptake of smPSH was 3.7- or 5.3-fold increased compared to that of PS or PTX solution. The AUC of smPSH was increased 3- or 1.2-fold compared to that of PTX solution or PS and the C_{max} was increased 2- or 1.1-fold compared to that of PTX solution or PS. These results implied smPSH increased the extent of absorbed PTX not the rate of absorption. Notably, the concentration of PTX in the lymph nodes at the final time point was recorded and the highest PTX amount was found in the smPSH group. The lymphatic uptake

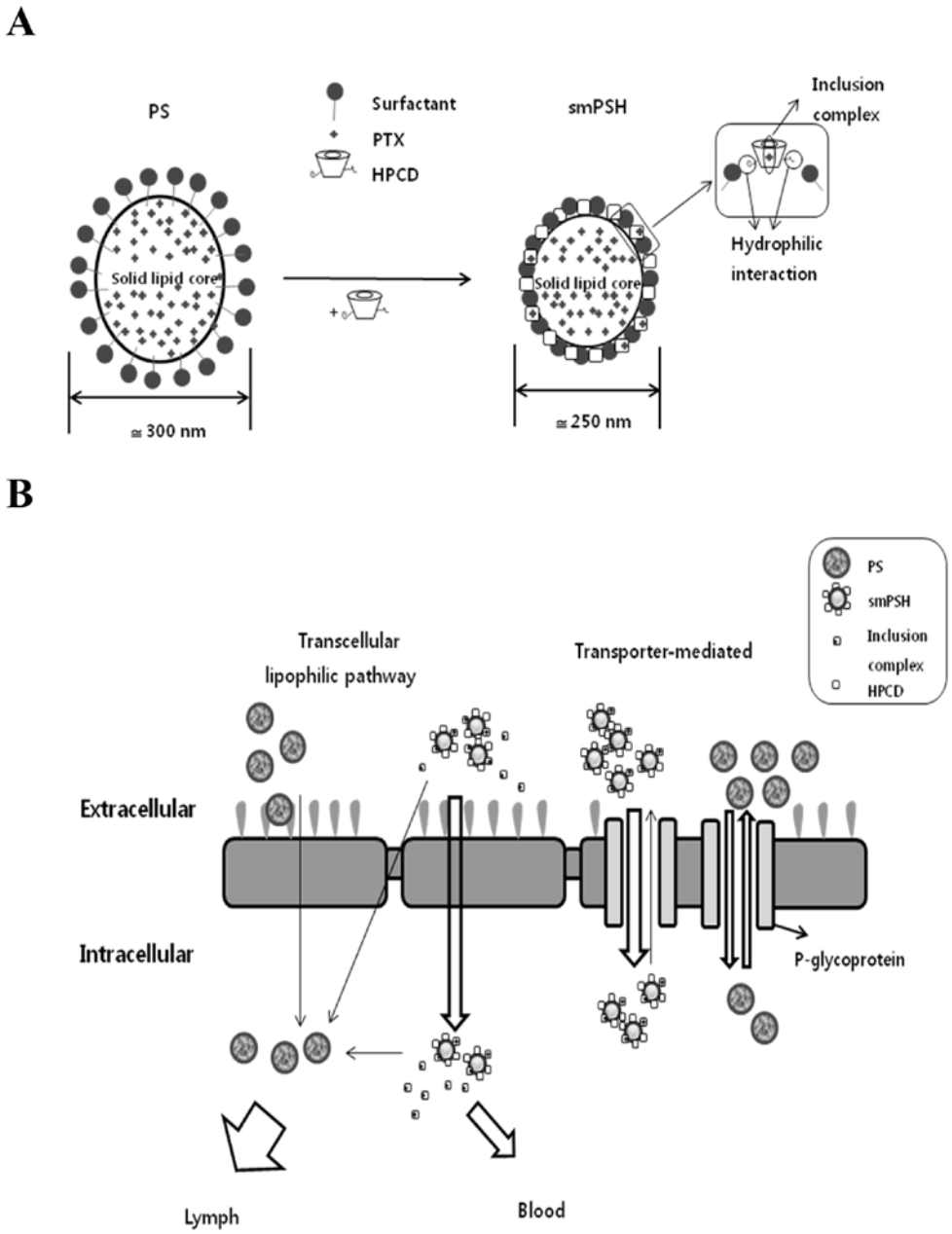

Figure 6. Schematic representation of (A) the formation process of smPSH and (B) the mechanism of cellular uptake.

of smPSH was increased 12.5- or 1.2-fold compared to PTX solution or PS, respectively. Recent advancements to improve bioavailability involves the utilization of the lymphatic circulation in oral delivery as it circumvents the hepatic first pass effect. It is known that lymphatic targeting can be achieved through lipid-based carrier systems such as microemulsion, nanoemulsion and SLNs (20). Taken together, it may be explained that smPSH increased the lymphatic uptake of PTX and consequently improved the bioavailability.

Based on these results, a schematic model was constructed (Fig. 6). First, it was observed that HPCD led to a decrease in the particle size of the nanoparticles of PTX and an increase in the hydrophilicity of PTX by observing an increase in solubility and dissolution of PTX in the form of smPSH. When HPCD was added to PS, PS was completely entangled by hydrophilic interaction between the hydrophilic part of HPCD and the hydrophilic head of the surfactant and then smPSH was formed. Thus, the particle size of PS (302.10 nm) was decreased to approximately 251.40 nm in smPSH. A part of PTX present outside PS entered into the pocket of HPCD during the formation of smPSH, and smPSH exhibited high encapsulation efficiency. In addition, the initial burst release may be explained by the PTX present at the pocket of HPCD in smPSH (Fig. 6A). Therefore, HPCD plays an important role in decreasing the particle size of the PTX nanoparticle and increasing its solubility and dissolution.

In regards to the safety of HPCD, a number of clinical studies are reported in the literature and have shown that HPCD is well tolerated and safe in the majority of patients receiving HPCD at daily oral doses of 4-8 g for at least 2 weeks (21). Higher oral daily doses of 16-24 g when administered for 14 days to volunteers resulted in increased incidences of soft

stools and diarrhea. Based on these clinical data, HPCD was considered to be non-toxic (at least for 14 days) when the daily dose is <16 g. Hence, HPCD was used for smPSH in this study.

Based on cellular uptake results, once smPSH was administered, smPSH entered into the cells by endocytosis. Simultaneously, HPCD was easily dissolved in the extracellular fluid, and PS was separately uptaken into the intracellular fluid by endocytosis. Subsequently, SLNs of PTX (PS) consisted of long-chain fatty acid such as stearic acid and more PTX was distributed into the lymphatic circulation (22) and PTX complexed into HPCD was distributed into blood circulation. Moreover, the p-glycoprotein (p-gp) transporter recognized PTX to a lesser extent since PTX existed in the form of smPSH, thus PTX could not be effluxed from the intracellular domain to the extracellular domain (Fig. 6B). P-gp is known to recognize and transport a variety of large, structurally and functionally unrelated, neutral or cationic anticancer drugs, including vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes (23). A common strategy to circumvent p-gp-based MDR is to co-administer a p-gp inhibitor along with anticancer drugs (24). Therefore, HPCD may contribute as a p-gp inhibitor.

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