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표적항암 약물전달체용 담즙산-히알루론산 유도체의 파클리탁셀 봉입 나노입자

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Paclitaxel-loaded Nanoparticles of Cholanic Acid-Modified Hyaluronan Oligosaccharide for Tumor-site Specific Delivery

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초록: 본 연구에서는 저분자량의 히알루론산에 5-β-cholanic acid를 conjugation하여 *in vivo*에서 종양표적성이 있고 투과율이 증가된 자기집합 나노입자를 제조하였다. 주사용으로 적합한 점도를 갖는 히알루론산 분자량은 16000 g/mol이었으며, 자기집합 나노입자를 만들 수 있는 cholanic acid의 히알루론산에 대한 최적 치환율(degree of substitution)은 14% 였다. 최적화된 파클리탁셀의 feed amount를 구하기 위해 파클리탁셀이 봉입된 나노입자의 크기와 안정성을 비교하였고 그 비는 30%가 적합하였다. 파클리탁셀이 봉입된 나노입자를 사용하여 biodistribution을 조사한 결과 HA-CA 나노입자의 혈액 내 long circulation 특성과 생체 내 종양표적 특이성으로 인하여 기존의 나노입자보다 간으로 분포되는 양이 현저히 줄어들었다. 또한 유방암 xenograft 모델을 이용한 항암효율 측정에서도 파클리탁셀이 봉입된 나노입자는 양성대조군보다 종양성장을 더 많이 저해함을 보여주었다.

Abstract: We have demonstrated that introduction of 5-β-cholanic acid into low molecular weight hyaluronic acid oligosaccharide produces self-assembled nanoparticles with enhanced permeability to a tumor site *in vivo*. We have found that hyaluronic acid of molecular weight 16000 g/mol has appropriate injectable viscosity. For making self-assembled nanoparticles with the selected hyaluronic acid, the optimal degree of substitution value of cholanic acid was 14%, which was selected by considering the size and stability of nanoparticles after loading with paclitaxel. 30% paclitaxel was selected as the optimal feed amount to make the paclitaxel-loaded hyaluronic acid-cholanic acid nanoparticles. Importantly, the optimal formulation of paclitaxel-loaded nanoparticles showed less liver uptake than the reported formula, along with long circulation as well as tumor-site specific accumulation in the *in vivo* biodistribution study. In the antitumor efficacy study, the paclitaxel-loaded nanoparticles showed higher efficacy to inhibit tumor growth than a positive control in the breast cancer cell xenograft model.

Keywords: hyaluronic acid oligosaccharide, self-assembled nanoparticle, paclitaxel, site specific delivery, antitumor efficacy.

Introduction

Hyaluronic acid (HA) has attracted much attention in tumor-targeted delivery because HA possesses numerous desirable physicochemical and biological properties including biocompatibility, biodegradability and non-immunogenicity for drug delivery applications.[1-5] The most widely distributed form of HA in normal tissues is high molecular weight (MW HA (~ 10^7 Da).[6] The lower MW forms of HA mediate degradation or oxidative hydrolysis of native HA under pathological conditions including repair, inflammation and tumor processes.[7,8] Lower MW HA can be differentiated from low MW HA (0.8 to 8×10^5 Da) and hyaluronan oligosaccharides (oHA) (<10^4 Da). HA can specifically bind to various cancer cells that overexpress

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CD44 receptor.[9,10] CD44 is a major cell surface receptor for HA and has an N-terminal link module homology domain that is responsible for binding to HA.

A number of groups have demonstrated that introduction of 5-β-cholanic acid (CA) into low MW HA (2.3×10^5 Da; HA_{230000}) can induce self-association to form self-aggregates and increase the ability of HA to effectively reach the tumor site *in vivo*.[11-14] The 5-β-cholanic acid-conjugated hyaluronic acid nanoparticles (HA_{230000}-CA NPs) were investigated for tumor therapy and imaging because of their tumor-targeting ability *in vivo*.[11,15] After systemic administration, these NPs effectively reached the tumor site based on the enhanced permeability and retention (EPR) effect as well as through an active targeting mechanism that involves the binding of HA to CD44, the HA receptor over-expressed on tumor cells.[16,17]

Although there was highly selective accumulation of HA_{230000}-CA nanoparticles in the tumor site, due to the high viscosity and swelling of HA even at low MW, the formula has not yet been developed into a final product: The molecular weight of HA used to make the reported HA-CA nanoparticles was approximately 234000 g/mol, which is still highly viscos when it is diluted to the calculated amount of HA-CA conjugates for an effective drug dose for humans.[14]

Another issue of the HA_{230000}-CA nanoparticles is low drug loading capacity.[2] The low loading amount of drug (Paclitaxel: PTX) may result in the need for a relatively high dose of polymer as a carrier and subsequently lead to administration issues including solubility in water or injectability of the resulting viscous solution as well as undesirable toxicity of polymer.[18] The low drug-loading capacity may come from the imbalance between hydrophobicity and hydrophilicity of the amphiphilic polymer as well as the electrostatic interaction between the drug and carrier. The conjugation ratio of the hydrophobic moiety to backbone hydrophilic polymer plays a significant role in the balance between the hydrophobicity and hydrophilicity of the polymer. As the degree of substitution (DS) value decreases, the integration of particles with hydrophobic core may decrease, resulting in low drug loading capacity.[12-14]

In this study, we chose oHA with MW in the range of 0.8-1.6×10^4 Da to solve the high viscosity issue. The optimal conjugation ratio of β-cholanic acid to oHA was determined to generate the PTX-loaded, self-assembled nanoparticles with desired efficacy. To enhance the loading capacity of paclitaxel, which is a hydrophobic cytotoxic agent, the optimal HA-CA conjugates were also selected. The feed amount of paclitaxel was determined by considering the particle size and stability of the nanoparticles. The optimally formulated paclitaxel-loaded HA-CA nanoparticles were evaluated to determine whether they could accumulate with high selectivity in the tumor-specific site and to assess their antitumor efficacy in tumor-bearing mice.

Experimental

Materials. Sodium hyaluronates (molecular weight 250000, 135000, 16000, 7500 g/mol) were purchased from Lifecore Biomedical (MN, USA). Paclitaxel (PTX) and Genexol® were obtained from SamYang Biopharm Co. (Seoul, Korea). 5β-cholanic acid, adipic acid dihydrazide (ADH), ethylenediamine, 1-hydroxybenzotriazole (HOBt), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich Co.(MO, USA). Cyanine 5.5 (Cy5.5), was obtained from Amersham Biosciences (NJ, USA). MDA-MB231 cells (Human breast cell carcinoma) were purchased from the American Type Culture Collection (MD, USA). All other reagents were analytical grade and used as received.

Preparation of Hyaluronic Acid-5-β-Cholanic Acid (HA-CA) Conjugates. Synthesis of Aminoethyl-5-β-Cholanoamides (EtCA): 5-β-Cholanic acid (10 g) was converted to its methyl ester, MtCA, by treating it with 1.6 mL hydrochloric acid in methanol (600 mL) at 60 °C for 6 h. MtCA was obtained by evaporation of methanol to give residue, which was dried by lyophilization and used for the next step. The obtained methyl ester of 5-β-cholanic acid was then treated with ethylenediamine (60 mL) and stirred at 130 °C for 8 h. After being cooled to room temperature, the desired product, EtCA, was precipitated by treating with 2 L distilled water. The precipitate was dried by lyophilization. A schematic diagram is shown in Figure 1.

Synthesis of HA-CA Conjugates: Hydrophobic EtCA with different molar ratio was chemically conjugated to the backbone of HAs (molecular weight 250000, 135000, 16000, 7500 g/mol) in the presence of EDC and NHS. A schematic diagram is shown in Figure 2.

Briefly, EDC (1.57 g) and NHS (0.941 g) were added to a solution of HA (1 g) in distilled water (160 mL) to generate the NHS-active ester of HA. This active ester solution was added dropwise to a solution of EtCA (125 mg) in dimethylformamide (DMF, 250 mL), and the resulting mixture was stirred at 60 °C for 3 days to afford the HA-CA conjugate. The HA-CA conjugate was precipitated by addition of a mixed solution of

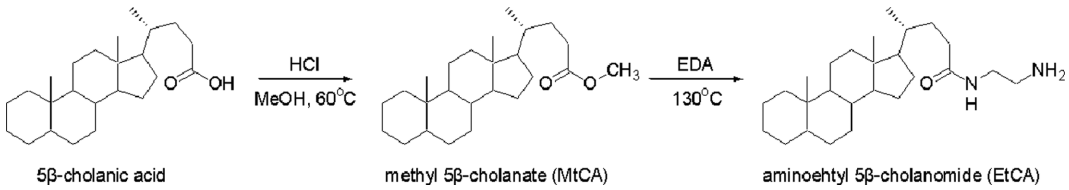

Figure 1. Schematic diagram of the conversion of 5-β, cholanic acid to EtCA (aminoethyl 5-β, cholanoamide).

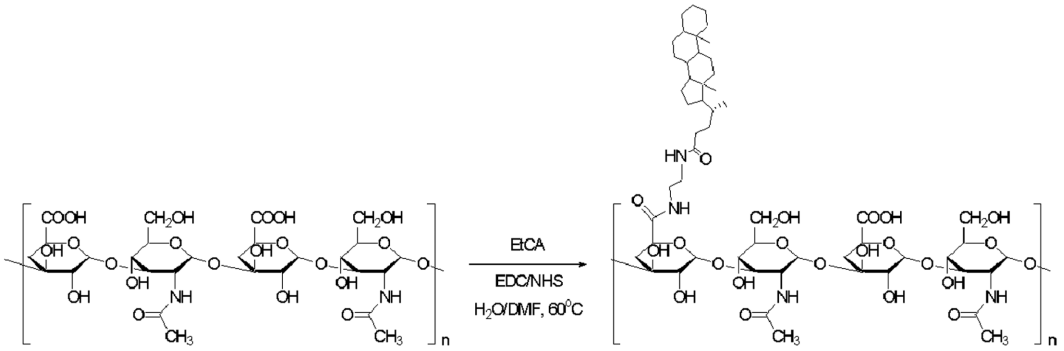

Figure 2. Schematic diagram of the synthesis of HA-CA conjugate.

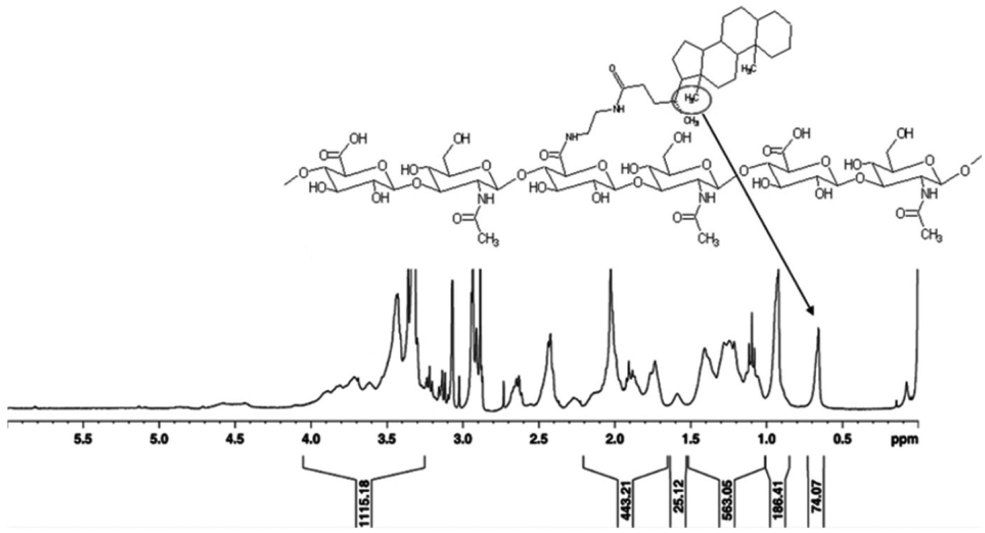

Figure 3. ^{1}H NMR spectra of HA-CA conjugates in D_2O/CD_3OD (1v/2v). Arrows indicate characteristic peaks of 5-β-cholanic acid conjugated to hyaluronic acid.

isopropyl alcohol and isopropyl ether (1:1, by volume). The precipitates were collected, washed with the mixed solution three times and dried by vacuum. By varying the molar ratio of EtCA to the carboxylic acid of HA, the degree of substitution (DS, defined as the number of EtCA molecules per 100 sugar residues of HA) was determined using ^{1}H NMR (UnityPlus 300, Varian, CA, USA) as shown in Figure 3, for which the sample was prepared by dissolving the conjugate in D_2O/CD_3OD (1/1, by volume).

Preparation of PTX-loaded HA-CA Nanoparticles: Paclitaxel (PTX)-loaded HA-CA nanoparticles were prepared by the film hydration method. Briefly, HA-CA conjugates (90 mg) were dissolved in distilled water (10 mL). A predetermined amount of PTX with different feed ratios was dissolved in 30 mL ethanol, then mixed with HA-CA conjugates solution. Thereafter, the solvent of the mixed solution was evaporated to create a thin film of the complex of PTX-loaded HA-CA conjugates using the rotary evaporator. The film was

hydrated with distilled water (50 mL), filtered through 0.8-μm syringe filter to remove non-loaded PTX crystals, and lyophilized. The loading amount of PTX was measured by isocratic reversed-phase HPLC using a 3100 series HPLC system (Agilent Technologies, Wilmington, DE) with a μ-Bondapak C18 column (150 mm L. ×4.6 mm I.D Waters, Milford, MA). The mobile phase consisted of methanol/water (80:20, by volume) delivered at a flow rate of 1.0 mL/min. Eluted compounds were detected at 240 nm using a Spectra100 UV-Vis detector.

Characterization of PTX-loaded HA-CA Nanoparticles. The mean diameter of PTX-loaded HA-CA nanoparticles were evaluated by DLS (dynamic light scattering) measurements using NICOMP 380ZLS (PSS NICOMP, Port Richey, Fl) operated at 633 nm and 25 °C.

Tumor Targetability and Biodistribution of PTX-loaded HA-CA Nanoparticles. NIH guidelines for the care and use of laboratory animal (NIH publication #85-23 Rev. 1985) were followed for all animal studies. For the *in vivo* biodistribution study, HA-CA conjugates labeled with an NIR dye, Cy5.5, were used to make PTX-loaded HA-CA nanoparticles and to visualize their biodistribution after injection into tumor-bearing mouse. In brief, HA-CA conjugates were chemically modified with ADH-modified Cy5.5 in the presence of EDC and HOBt. The content of Cy5.5 molecules in the conjugate was fixed to 3.2 wt%, as determined with a UV/VIS spectrophotometer at 680 nm. As mentioned above, PTX was loaded into the Cy5.5-labeled HA-CA conjugates. Approximately 1×10^6 MDA-MB231 cells in physiological saline (100 μL) were injected subcutaneously into the tumor-bearing nude mice (mice aged 7 weeks). To observe biodistribution and tumor accumulation, Cy5.5-loaded HA-CA nanoparticles were injected into the tail vein of the tumor-bearing mice at a dose of 5 mg/kg after fourteen days of subcutaneous inoculation. The eXplore Optix system (ART Advanced Research Technologies Inc., Montreal, Canada) was used to obtain the image as previously described protocol.[11]

Tumor Regression Study on PTX-loaded HA-CA Nanoparticles. Xenograft Balb/c male mice were utilized to evaluate tumor regression by PTX-loaded HA-CA nanoparticles. In the subcutaneous model, 1×10^6 MDA-MB231 cells in 100 μL saline were inoculated to create a dorsal lesion (mice aged 7 weeks) and were allowed to grow into tumors 8 mm in diameter. Seven days after subcutaneous inoculation, Balb/c male mice were treated with i.v. tail injection of HA-CA nanoparticles containing 16% PTX, Genexol® (SamYang Biopharm), or saline. The survival and the body weight of the mice were measured. Tumor regression was assessed with tumor volume, quantified by $a\times b^2/2$, where a is the longest tumor diameter and b is the shortest tumor diameter. The differences between experimental and control groups were analyzed using one-way ANOVA and considered statistically significant [marked with an asterisk (*) in figures] if $p<0.05$.

Results and Discussion

Characteristics of HA-CA Conjugates. HA-CA conjugates were prepared by reaction of 5-β-cholanic acid and a

Table 1. Effect of Feed Ratio on the Degree of Substitution (DS) and Solubility of Self-aggregates

Sample	Molecular weight of HA	Feed ratio (%)	DS Value (%)	Solubility (g/mL)	Efficiency of DS (%)
HA-CA1	7K	10	8	80	80
HA-CA2	7K	20	Not soluble in any solvent	-	-
HA-CA3	16K	10	8	-	80
HA-CA4	16K	20	10-12	-	80
HA-CA5	16K	30	14	50	47
HA-CA6	16K	40	Not soluble in any solvent	-	-
HA-CA7	135K	10	9	-	90
HA-CA8	135K	30	25	25	83
HA-CA9	135K	40	Not soluble in any solvent	-	-
HA-CA10	250K	10	9	-	90
HA-CA11	250K	30	27	-	90
HA-CA12	250K	40	30	10	75

water-soluble HA with different molecular weight according to previous description.[11] As shown in Table 1, the degree of substitution (DS) values of EtCA varied depending on the molecular weight of HA and the feed ratio of EtCA.

The DS efficiencies of HA-CA1 and HA-CA3 with oHA were not significantly different from HA-CA7 and HA-CA10 with higher MW HA. However, the higher feed ratio of EtCA could make the bigger difference in DS efficiency between oHA and higher MW HA. DS value is the key factor for the hydrophobicity of HA-CA conjugates and for their water solubility. As shown in Table 1, the maximum DS values were determined by the solubility and the self-assembling capability in water. DS values greater than the maximum resulted in water-insoluble HA-CA conjugates due to the high hydrophobicity, therefore, they were not utilized. We found that the lower MW HA required a smaller feed ratio of EtCA to produce amphiphilic characteristics and in detail, HA-CA1, HA-CA5, HA-CA8, and HA-CA12 had the optimal DS values to make nanoparticles. HA-CA8 and HA-CA12 were not adequate for injection because of their high viscosity due to high MW HA. Consequently, HA-CA1 and HA-CA5 were chosen as optimal HA-CA nanoparticles for loading PTX.

PTX-loaded HA-CA Nanoparticles. Paclitaxel-loaded HA-CA nanoparticles were prepared by the film hydration method, and particle sizes were measured by DLS. Based on the particle size measurements, the optimal HA-CA conjugates (HA-CA1 or HA-CA5) and the loading amount of PTX (10, 20, 30 or 40%) were determined. Overall, the mean diameter of PTX-loaded HA-CA nanoparticles ranged from 250-340 nm and showed a narrow size distribution. In the case of HA-CA1, the mean diameters varied depending on the loading amount of PTX as shown in Figure 4.

10% PTX-loaded HA-CA nanoparticles had approximately 700 nm bigger particle size comparing to 20% or 30% PTX-loaded HA-CA nanoparticles. As the loading amount of paclitaxel increased, the particle size may decrease due to the formation of a more compact hydrophobic inner core. 40% PTX was readily released and crystallized from the nanoparticle. In

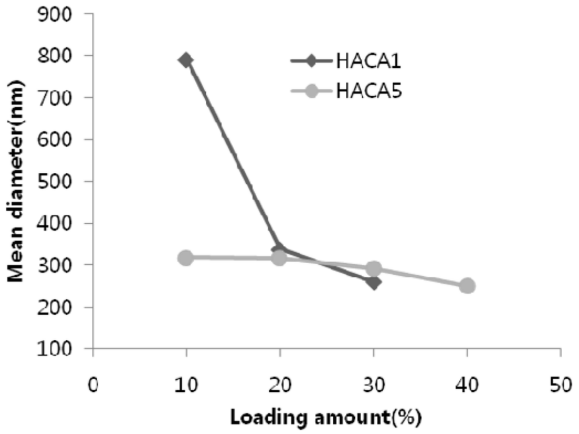

Figure 4. Changes in particle sizes of the low MW HA depending on the loading amount of PTX. (◆): HA-CA1 (7 K HA-10%CA), (●): HA-CA5 (16 K HA-30%CA) The particle sizes were determined by dynamic light scattering (DLS).

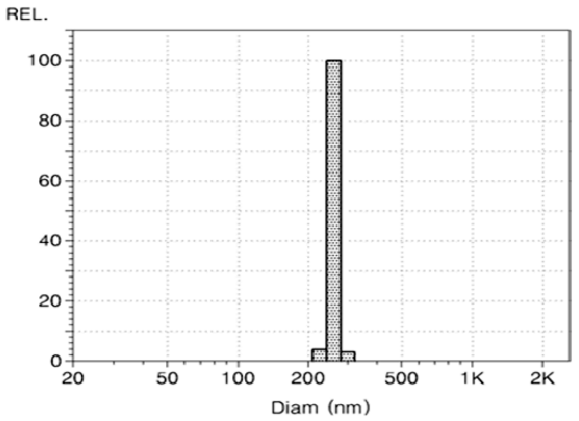

Figure 5. Size distribution of paclitaxel-loaded HA-CA nanoparticles as determined by dynamic light scattering (DLS).

HA-CA5, 10, 20, 30 or 40% paclitaxel was stable and nanoparticle ranged from 260-319 nm in size as shown in Table 2.

We selected HA-CA5 as the optimal conjugate with respect to the loading amount of paclitaxel, particle size and water solubility. The loading amount and efficiency of PTX-loaded HA-CA5 were determined by HPLC to select the optimal feed amount of PTX. Considering the loading efficiency and

Table 2. Effect of Feed Ratio of PTX on Loading Amount, Loading Efficiency and Mean Diameter

Sample of HA-CA conjugate	Feed ratio of paclitaxel (%)	Loading amount (%)	Loading efficiency (%)	Mean diameter (nm)
HA-CA5	10	8.8	88.2	318.4±35
	20	16	79.6	316.4±76.8
	30	25.6	85.2	292.4±84.5
	40	31	77.5	250.5±72

amount of PTX, 30% PTX is the optimal feed amount for HA-CA nanoparticles. Figure 5 shows that PTX-loaded HA-CA nanoparticles have a narrow size distribution.

In this study, we demonstrated that HA-CA conjugates can form self-assembled nanoparticles with 25% loading amount of drug, which formula has great potential for clinical application.

In vivo Biodistribution of PTX-loaded HA-CA Nanoparticles. To investigate *in vivo* biodistribution of PTX-loaded HA-CA nanoparticles, a non-invasive near infrared optical imaging technique was used in tumor-bearing mice. HA-CA conjugates with different MW HA were labeled with the NIR fluorophore Cy5.5, and then PTX-loaded HA-CA nanoparticles were produced. The Cy5.5-labeled PTX-loaded HA-CA nanoparticles were intravenously administered into breast cancer tumor-bearing mice (5 mg/kg, nanoparticles/mice). Figure 6 shows the fluorescence signals of Cy5.5 of nanoparticles in the tumor-bearing mice.

We observed an accumulation of Cy5.5 in tumor but reduction in the whole body over two days, resulting from circulation of PTX-loaded HA-CA nanoparticles in the bloodstream. HA-CA1 (MW of HA: 7000 g/mol) showed faster discrimination than HA-CA5 (MW of HA: 16000 g/mol) nanoparticles, which exhibited the strongest fluorescent signal at the tumor tissue.

Anti-tumor Efficacy of PTX-loaded HA-CA Nanoparticles. In order to test the effects of PTX-loaded HA-CA or Genexol® (as control) on tumor growth *in vivo*, xenograft model of Balb/c male mice were studied. These animals were administered intravenously through the tail (injections at 0, 3, 7 days). The dose of Genexol® was 20 mg/kg, which is the maximum tolerated dose.[21] In the experimental group, paclitaxel-loaded HA-CA nanoparticles at doses corresponding to 20 and 40 mg/kg were used because these formulations were less cytotoxic than Cremophor EL formulations (Genexol®). Mice were also injected with 200 μL of saline as controls. Tumor growth was significantly inhibited by treatment with paclitaxel-loaded HA-CA nanoparticles compared with saline as shown in Figure 7.

PTX-loaded HA-CA nanoparticles showed similar *in vivo* anti-tumor activity compared with Genexol® as a positive control after 3 days. Tumor growth inhibition was more efficient in mice treated with 40 mg/kg PTX-loaded HA-CA nanoparticles than in mice treated with 20 mg/kg PTX-loaded HA-CA conjugate or Genexol®. Table 3 shows that PTX-loaded HA-CA nanoparticles had greater efficacy to inhibit tumor growth than paclitaxel-loaded HA-CA nanoparticles Genexol®.

The groups that received PTX-loaded HA-CA nanoparticles showed complete inhibition of tumor growth at day 32 compared with the positive control group that received Genexol, which showed re-growth of tumor tissue from day 27. Figure 8 shows the changes in the body weight of tumor-bearing mice after injection of the different PTX formulations.

Overall, there were no significant reductions in body weight

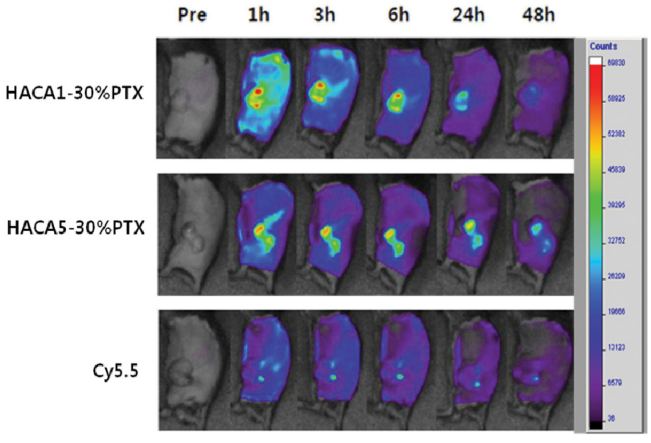

Figure 6. *In vivo* non-invasive NIR fluorescence images of real-time tumor targeting characteristics of paclitaxel-loaded HA-CA nanoparticles. *In vivo* fluorescence imaging of Balb/c mice bearing breast cancer cell tumors after intravenous injection of paclitaxel-loaded HA-CA nanoparticles.

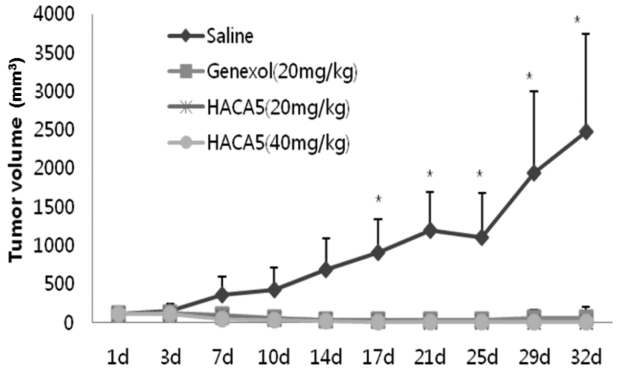

Figure 7. Therapeutic efficacy of paclitaxel-loaded HA-CA nanoparticles in MDA-MB231 breast cancer cell xenograft. The antitumor effects of paclitaxel-loaded HA-CA nanoparticles were determined by measurement of tumor size for 32 days.When initial tumor volumes were 50 to 70 mm^3, tumor-bearing mice (n=6) were intravenously injected in the tail with HA-CA conjugates containing 30% paclitaxel, Genexol, or saline at 0, 3, 7 day. The symbols are as follows: Saline (◆); Genexol (■); HA-CA5 (20 mg/kg) (●); HA-CA5 (40 mg/kg) (*). *indicates difference at the $p<0.05$ significance level.

Table 3. Relative Tumor Volume (RTV) in MDA-MB231 Breast Cancer Cell Xenograft Model. Relative Tumor Volume (RTV) is Calculated by Dividing the Tumor Volume at Any Time by the Tumor Volume at the Start of Treatment

(unit: %)

% of Start	Start	3 day	7 day	10 day	14 day	17 day	21 day	25 day	29 day	32 day
Saline	100	135	323	380	619	818	1074	998	1745	2235
Genexol PM (20)	100	107	88	46	33	28	27	22	46	54
HA-CA5 (20)	100	95	59	39	30	12	10	0	3	7
HA-CA5 (40)	100	101	45	23	17	6	3	2	4	1

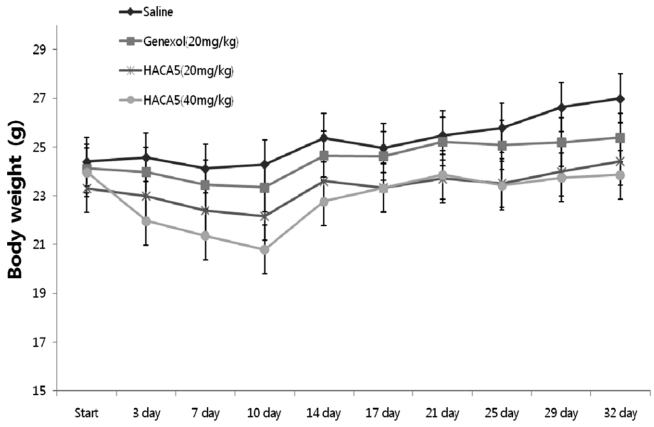

Figure 8. Body weight changes of mice receiving paclitaxel-loaded HA-CA nanoparticles. The symbols are as follows: Saline (◆); Genexol (■) HA-CA5 (20 mg/kg) (●); HA-CA5 (40 mg/kg) (*).

in any group. In the group that received 40 mg/kg of PTX-loaded HA-CA nanoparticles, there was a significant, approximately 10%, reduction in body weight compared to the group that received Genexol. At day12 after injection, the body weight was recovered and did not show a significant reduction compared to the positive control group.

In this study, we used oHA (0.8-1.6×10^4 Da) to solve the high viscosity problem of HA. The MW of HA ranges from hundreds of thousands up to several millions of Daltons.[22] The aqueous solutions of such high MW HA exhibit shear-dependent viscosity, and ultrapure viscous HA solutions have been introduced among preparations for various purposes, including as a 'viscosurgery' aid during operations of the anterior chamber of the eye (Healon) and as a 'viscosupplementing' tool administered intra-articularly into the osteoarthritic knee joint.[23] For the medical applications mentioned above, the MW of HA is higher than 10^6 Da. In this study, 1.35×10^5 Da HA or above was found to be too viscous to be intravenously injected into tumor-bearing mice at the calculated concentration of polymer with the human-effective dose of drug. In initial studies of hydrophobically modified HA with cholanic acid, 234000 g/mol MW HA was used to make self-assembled nanoparticles (HA-CA nanoparticles).[2,11] The results from the study demonstrated that all HA-CA nanoparticles selectively accumulated in the tumor site and significant fluorescent signals were detected in dissected tumors after 48 h. Based on these results, we tried to make PTX-loaded HA-CA nanoparticles and evaluate whether they have antitumor efficacy in the breast cancer cell xenograft. However, we found that 234000 g/mol MW HA was too viscous to be injected intravenously with 20 mg/kg paclitaxel. The amount of HA-CA used could be calculated as almost 200 mg/kg if the loading amount of drug in HA-CA was assumed to be approximately 10% and by considering the injection volume (5-10 mL/kg); the HA-CA nanoparticles should thus be diluted to a concentration of 20-40 mg/mL, which is still highly viscous and is not proper for injection. Even though PTX is injected via infusion in the clinic, highly viscous nanoparticles may induce inflammation, circulate too long in the blood or be difficult to be excreted. We chose oHA with MW of 0.8~1.6×10^4 Da as an optimal HA based on injectability at the diluted concentration of 20-40 mg/mL HA-CA in saline or distilled water. The oHA is readily soluble in water.

To enhance the loading capacity of PTX, the conjugation ratio of hydrophobic moiety is an important factor, which balances the hydrophobicity and hydrophilicity of a polymer. We found that the lower MW HA required the smaller DS value of EtCA to exhibit amphiphilic characteristics. For self-assembled nanoparticles with 16000 g/mol MW HA, the optimal degree of substitution value of cholanic acid was 14%, which was selected by considering the water solubility and self-assembly capability. The feed amount of PTX was determined by considering the particle size and stability of nanoparticles. Self-assembled oHA-CA may have lower stability than nanoparticle with higher MW HA because self-assembled oHA-CA may form micelles rather than nanoparticles or aggregates. The optimal conjugation ratio of CA to oHA that we found in this study could balance the hydrophillicity and hydrophobicity of

HA-CA and make the high loading amount (approximately 25%) of PTX possible. The optimally formulated PTX-loaded HA-CA nanoparticles were evaluated to assess their selective accumulation at the tumor site and their antitumor efficacy in tumor-bearing mice. The optimal formulation of PTX-loaded HA-CA nanoparticles showed less liver uptake than the reported formula, along with long circulation as well as tumor-specific accumulation in the *in vivo* biodistribution study. Liver uptake, which may be a major disadvantage of HA by intravenous injection, was not observed in this experimental study. In other studies, the PTX-loaded HA-CA nanoparticles showed considerable signals in the liver, which may be due to cellular uptake of the PTX-loaded HA-CA nanoparticles by phagocytic cells of the reticuloendothelial system and by liver sinusoidal endothelial cells expressing the HARE receptor.[19,20] Our results may indicate that the tightly integrated PTX-loaded HA-CA nanoparticles have less liver uptake while maintaining the promising properties of other nanoparticles based on the higher MW HA: the long circulation and accumulation at the specific site of the tumor. When we tested three samples, HA-CA1, HA-CA5, and Cy5.5, HA-CA5 exhibited the strongest fluorescent signal at tumor site (Figure 6).

In the antitumor efficacy study, PTX-loaded HA-CA nanoparticles had higher efficacy for tumor growth inhibition than Genexol in the breast cancer cell xenograft model. Tumor growth inhibition was more efficient in mice treated with 40 mg/kg PTX-loaded HA-CA nanoparticles than in mice treated with 20 mg/kg PTX-loaded HA-CA conjugate or Genexol® (Figure 7). The groups that received PTX-loaded HA-CA nanoparticles showed complete inhibition of tumor growth at day 32 compared with the positive control group that received Genexol. Genexol- received group showed re-growth of tumor tissue from day 27 (Table 3).

The optimal formulation of PTX-loaded HA-CA nanoparticles was determined based on the viscosity of HA, the ease of use including resuspension time and loading amount of drug, the selective accumulation in tumor tissue, and the high efficacy for tumor growth inhibition in tumor-bearing mouse. The mechanism by which PTX-loaded HA-CA nanoparticles cause tumor regression is not currently clear, but we speculate that large amounts of PTX-loaded HA-CA nanoparticles accumulated in the tumor tissue as shown in Figure 6 and affected the tumor growth by changing the local environment in the tumor tissue. Choi *et al*. demonstrated that, after systemic administration, HA nanoparticles can effectively reach the tumor site based on the enhanced permeability and retention (EPR) effect, as well as through an active targeting mechanism through binding of HA to CD44, the HA receptor over-expressed on tumor cells.[16,17]

Conclusions

In this study, we demonstrate that an oligomer form of hyaluronic acid (HA, molecular weight of 0.8~1.6×10^4 Da) formed self-assembled nanoparticles by conjugation of cholanic acid (CA). We found the optimal conjugation ratio of cholanic acid to produce the optimal formulation. The HA-CA conjugates had the ability to load up to 25% paclitaxel, which is a high loading amount. The optimal formulation of paclitaxel-loaded HA-CA nanoparticles showed less liver uptake and long circulation as well as tumor-specific accumulation in the *in vivo* biodistribution study. In the antitumor efficacy study, paclitaxel-loaded HA-CA nanoparticles had higher efficacy to inhibit tumor growth compared with Genexol in the breast cancer cell xenograft model. We therefore expect that the new formula of drug-nanoparticle based on the oHA has great potential to be developed as a commercially available nanocarrier.

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