Research Paper

Heparin–Paclitaxel Conjugates Using Mixed Anhydride as Intermediate: Synthesis, Influence of Polymer Structure on Drug Release, Anticoagulant Activity and *In Vitro* Efficiency

Ying Wang,[1,2,3] Dingcheng Xin,[1,2] Kaijian Liu,[1,2] and Jiannan Xiang[1,2,3,4]

Purpose. The heparin–paclitaxel conjugates using amino acid as linker (*HD2*), with low anticoagulant activity, the similar anticancer activity as paclitaxel, offer great potential for further investigation.

Methods. Two types of heparin–paclitaxel conjugates (*HD*) have been developed, in which *O*-acetylated heparin as carrier conjugates with paclitaxel by direct ester bond (*HD1*) and by inserting different amino acids as spacers, including valine, leucine, and phenylalanine (*HD2a*, *HD2b*, and *HD2c*), respectively. Specifically, mixed anhydride groups of carrier as activating intermediates mediate the synthesis of prodrugs. The *HD* conjugates are characterized by ^{1}H NMR, FT-IR and GPC. The percentage weight of drug and hydrolysis rate for *HD* are detected by UV and HPLC. The anticoagulant activity and cell cycle of MCF-7 of *HD* are measured by APTT and FCM, respectively.

Results. *HD2* conjugates show better solubility and faster hydrolysis rates than those of *HD1*. Meanwhile, the anticoagulant activity of *HD* is reduced and FCM analysis show that MCF-7 cells treated with *HD* are arrested in the G2/M phase of cell cycle.

Conclusions. Amino acids as linkers between paclitaxel and carrier are appropriate to facilitate the release of paclitaxel from carrier. Mixed anhydrides mediate the synthesis of prodrugs and *HD2* conjugates are expected to further investigate *in vivo* experiment.

KEY WORDS: amino acid as spacer; anticoagulant activity; hydrolysis rate; *in vitro* efficiency; mixed anhydride.

INTRODUCTION

Paclitaxel has shown significant antineoplastic activity against various human cancers. However, when clinically used, solubility and toxicity are two major problems (1). Previous attempts have been largely limited to design of small molecule paclitaxel derivatives, such as ester paclitaxel derivatives and phosphate paclitaxel derivatives, etc (2–5). In recent years, use of macromolecules for the targeted delivery of anticancer agents has generated considerable interest regarding enhancing therapeutic efficacy and reduc-

ing systemic side effects, and some satisfactory results have been obtained (6–9). Apparently, it would have been desirable to develop drug delivery systems which would be more soluble than paclitaxel, but which, upon hydrolysis under physiological conditions, would exhibit the same or similar level of antitumor activity as free paclitaxel. Furthermore, the rate of hydrolysis should facilitate the pharmacokinetics of the drug so as to enhance its delivery efficiency.

Heparin is a biocompatible, biodegradable and water-soluble natural polysaccharide with a complicated structure and rich in animal tissues (Fig. 1). Heparin has attracted intensive attention because it demonstrates a variety of biological activities, such as anticoagulant activity, inhibition of angiogenesis, tumor development, and of proliferation of arterial smooth muscle cells, and so forth (10,11). However, heparin therapy is possibly limited to use due to the risk of severe hemorrhagic complication. Heparin derivatives with low anticoagulant potency were prepared and applied to administrate *in vivo*. It was reported that heparin derivatives with reduced anticoagulant activity had a great potential as a drug on the tumor research (12).

It has been previously reported the preparation of ternary heparin conjugate by direct chemical bond strategy, and yielded the ternary conjugate with the reduced anticoagulant activity (W. Ying *et al.*, submitted manuscript). Importantly, we synthesized *O*-acetylated heparin with active mixed anhy-

[1] Biomedical Engineering Center, Hunan University, Changsha, 410082, China.

[2] College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, China.

[3] State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan University, Changsha, 410082, China.

[4] To whom correspondence should be addressed. (e-mail: jnxiang@hnu.cn).

Heparin :

major sequence   variable sequence

X=H or SO_3^-
Y=$COCH_3$ or SO_3^- or H
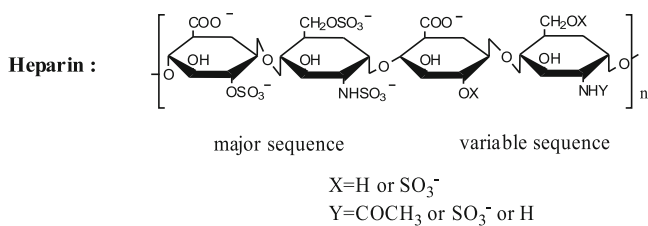
Fig. 1. Structure of heparin.

dride, which can facilitate the formation of prodrugs. Consequently, the ternary heparin conjugates by direct covalent bond strategy can be further extended to tumor research.
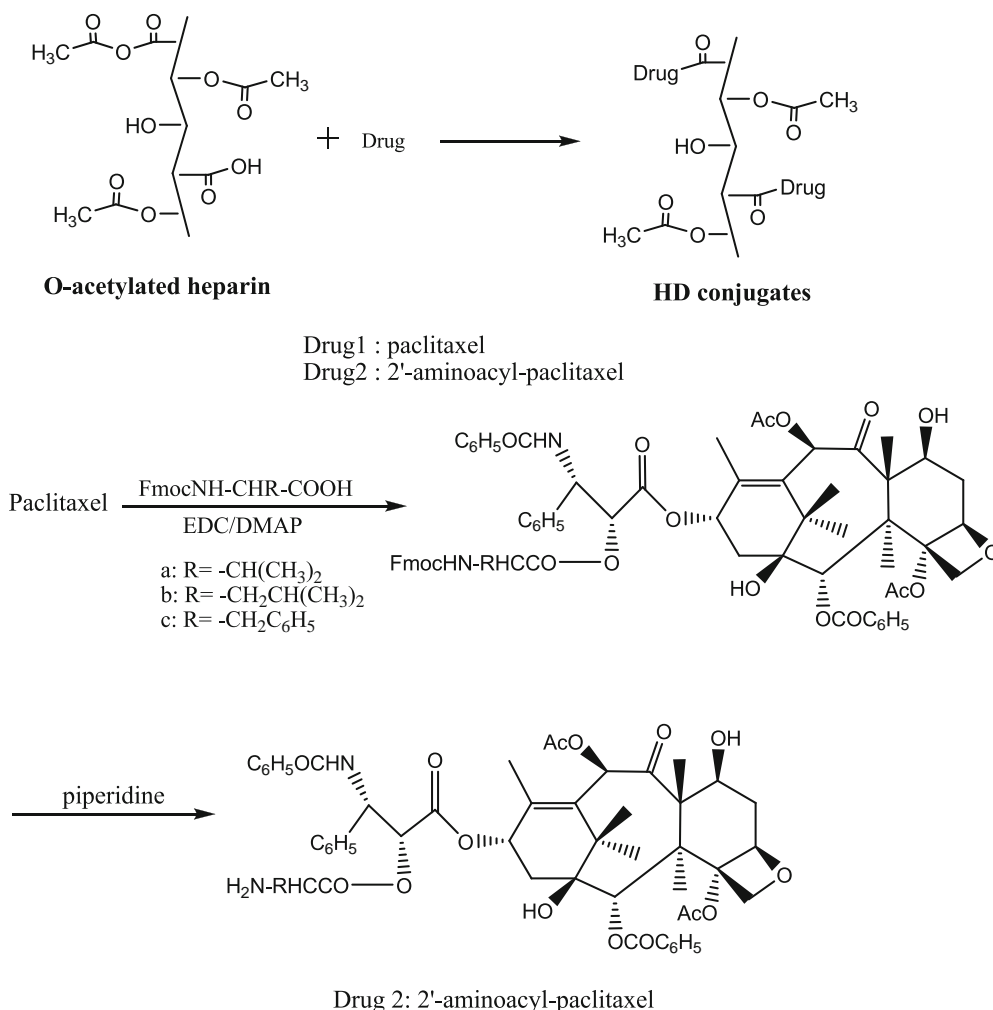
In the present study, we design and synthesis two drug delivery systems (*HD*), in which *O*-acetylated heparin conjugate with paclitaxel by direct ester bond (*HD1*) and by inserting amino acids as spacers, including valine, leucine, and phenylalanine (*HD2a*, *HD2b*, and *HD2c*), respectively. Previously reported 2'-aminocyl paclitaxel derivatives are quite unstable and readily revert to paclitaxel (13). Therefore, we investigate the hydrolysis rate and *in vitro* efficiency for *HD* conjugates. Besides, we try to examine whether conjugation of paclitaxel to heparin through the different amino acid

spacers would influence the pharmaceutical activity as compared with native paclitaxel. The designed synthetic route of the prodrugs (*HD* conjugates) is as follows (Fig. 2):

MATERIALS AND METHODS

Materials

Heparin (sodium salt, from porcine intestinal mucosa, $M_n=12$ kDa, 150 U/mg), *N,N*'-dimethylformamide (DMF), perchloric acid, piperdine, tributylamine, were purchased from Sinopharm chemical reagent Co.(Shanghai, China), and paclitaxel came from Tecano Science and Technology Co., Ltd. (Guangzhou, China). 2'-Valyl-paclitaxel, 2'-leucyl-paclitaxel, and 2'-phenylalanyl–paclitaxel were prepared in our lab. Esterase came from Sigma. Sephadex G-25, and dialysis membrane (MWCO 3,500) were purchased from Pharmacia (Piscataway, NJ, USA), and Viskase Co., Inc. (IL, USA), respectively. Reaction solvents were purified by distillation under nitrogen prior to use. Ultrapure water (Milli-Q, 18 M Ω) was used in the experiment. The MCF-7 (breast carcinoma) cell line was kindly donated by the Institute of Life Science and Biotechnology in Hunan University.

O-acetylated heparin   **HD conjugates**

Drug1 : paclitaxel
Drug2 : 2'-aminoacyl-paclitaxel

Paclitaxel $\xrightarrow[\text{EDC/DMAP}]{\text{FmocNH-CHR-COOH}}$

a: R= -$CH(CH_3)_2$
b: R= -$CH_2CH(CH_3)_2$
c: R= -$CH_2C_6H_5$

Drug 2: 2'-aminoacyl-paclitaxel

Fig. 2. Synthetic route of the prodrugs.

Synthesis

O-acetylated heparin. The preparation of *O*-acetylated heparin was described previously (W. Ying *et al.*, submitted manuscript). Briefly, heparin sodium salt (0.25 g) was dissolved in 20 mL of water and then percolated through a column (100 mL) of 732 (H$^+$) cation-exchange resin at 4°C. The pH of the solution was adjusted to 6.0 by addition of tributylamine (2 mL). Excess tributylamine was eliminated upon evaporating and a concentrated tributylammonium heparin salt was obtained. After lyophilizing, tributylammonium heparin salt (0.5 g) was obtained. The tributylammonium salt (0.5 g) was dissolved in dry DMF (10 mL) and cooled down to 0°C under nitrogen atmosphere. Then, acetic anhydride (1.33 g, 13 mmol), triethylamine (1.31 g, 13 mmol), and DMAP (0.036 g, 0.3 mmol) were successively added and the reaction was continued at room temperature for 24 h under nitrogen atmosphere. After cooling down to 0°C, cold ethanol (500 mL) was added. The precipitate was collected, dissolved in water, and passed through a column (100 mL) of 732 (H$^+$) cation-exchange resin at 4°C. Then the product was further purified by passing through a Sephadex G-25 column equilibrated in deionized water. The effluent was neutralized with 1 mol/L NaOH and lyophilized. At last, product 1a (0.14 g) was obtained as a white powder.

HD1. *O*-acetylated heparin (0.14 g) was reacted with paclitaxel (0.06 g, 0.072 mmol) using catalytic amount of HClO$_4$ as catalyst in dry DMF (20 mL) while stirring. The reaction was continued for 12 h at room temperature under nitrogen atmosphere. Excess DMF was evaporated under reduced pressure, and the filtrates were dissolved in water (5 ml) and then dialyzed against deionized water for 48 h using a dialysis membrane. After lyophilization, (0.18 g) was obtained as a white powder.

HD2a-c was synthesized as described for method of *HD1*.

Characterization

The ^{1}H NMR spectra of carrier and *HD* conjugates were recorded on a Varian INOVA400 apparatus in D$_2$O, CD$_3$OD and DMSO-d$_6$, respectively. FT-IR spectra were determined by a FD-5DX infrared spectrum apparatus. In addition, the molecular weight of *HD* conjugates was determined using a 515 Waters GPC system equipped with a Waters Ultrahydrogel 250 Column, a Waters 410 refractive index detector. 0.1 mol/L NaCl was used as an elution solvent at a flow rate of 0.6 mL/ min, and the column temperature was maintained at 40°C using polyethylene glycol standards.

UV spectra were obtained on a Techcomp UV-2300 Spectrophotometer (Shanghai, China). The percentage weight of drug on *HD* conjugates was estimated by UV measurements based on a standard curve generated with known concentrations of paclitaxel in ethanol ($\lambda=228$ nm).

In Vitro Release of Paclitaxel from HD Conjugates

The *in vitro* release of paclitaxel was characterized as described previously. Briefly, *HD* conjugates were dissolved in phosphate-buffered solutions (PBS, 0.01 M) at pH 7.4 or pH 5.0 and esterase (16 u/mL) in PBS (0.01 M) at pH 7.4 or pH 5.0 at an equivalent paclitaxel concentration of 1 g/L. The solutions were incubated at 37°C with gentle shaking. At selected time intervals, aliquots (500 μL) were removed and an equivalent PBS concentration was added to the solutions. The aliquots were extracted by chloroform and the organic fraction was evaporated. At the same time the residue mixed with acetonitrile (1,000 μL) and analyzed by HPLC.

The HPLC system consisted of a reverse-phase silica column (SepaxHP-C18, 4.6×250 mm, 5 μm, Sepax, USA), a mobile phase of acetonitrile and 0.05% trifluoroacetic acid in water (60:40) pumped (LC-20AT, Shimadzu) at a flow rate of 1.0 mL/ min. A 20 μL-aliquot of samples was injected and the column effluent was detected at 227 nm with a UV detector (SPD-20A, Shimadzu).

Anticoagulant Activity Assay

Anticoagulant activity of *O*-acetylated heparin and *HD* conjugates, using heparin as a standard, is evaluated by measuring activated partial thromboplastin time (APTT). The APTT was recorded on a PRECIL C2000 Coagulometer (Beijing, China). The reagents for the measurement of APTT were APTT (Ellagic acid) kits (Sun Biotech, China). The APTT was measured according to the manufacturer's procedure by using normal human plasma. Heparin derivative or
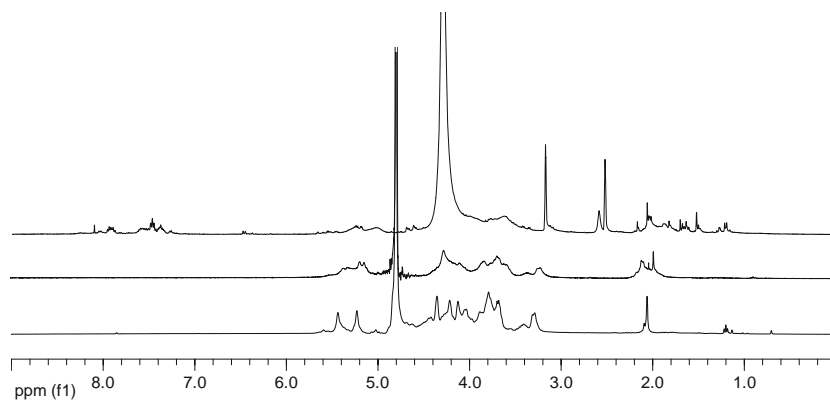
Fig. 3. ^{1}H NMR spectra of (*a*) Heparin, (*b*)Heparin derivative in D$_2$O and (*c*) *HD1* in DMSO-d$_6$ and CD$_3$OD (1:2).
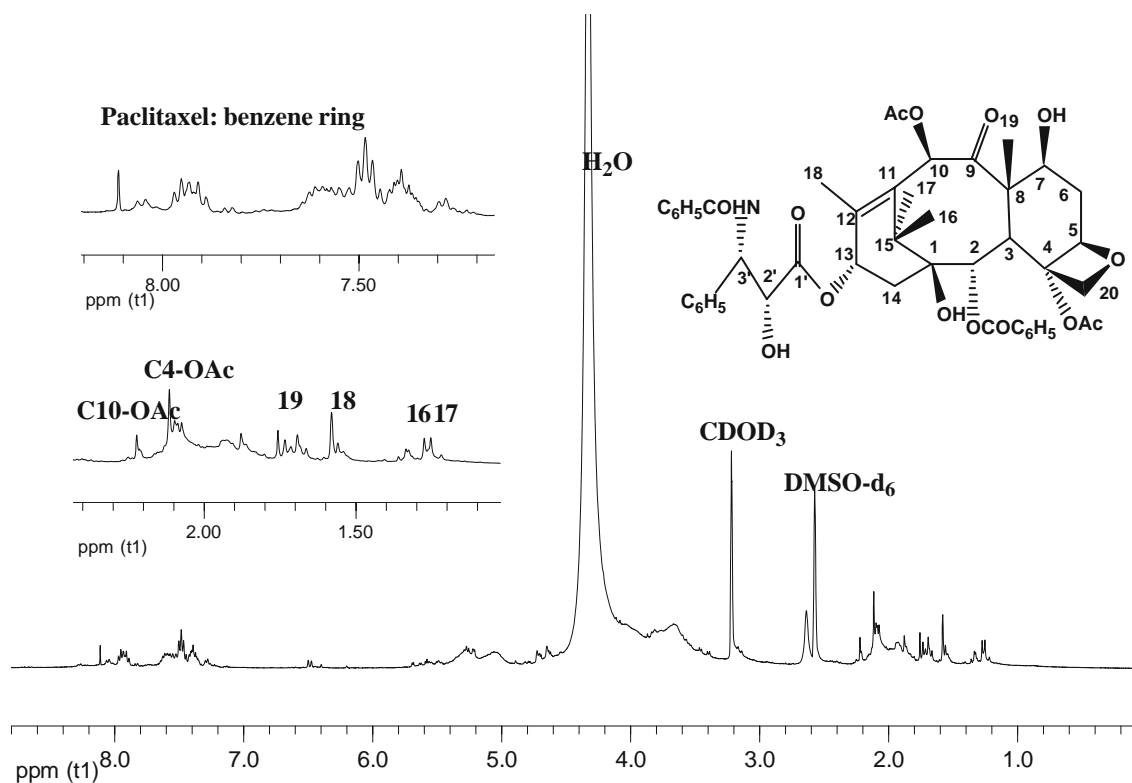
Fig. 4. ^{1}H NMR spectra of *HD1* in DMSO-d$_6$ and CD$_3$OD (1:2).

HD conjugates (2.5 μg/mL, 25 μL) was added to 0.1 mL of citrated plasma, followed by adding 0.1 mL of APTT reagent. This mixture was incubated at 37°C for 2 min. Then, pre-warmed CaCl$_2$ solution (0.1 mL, 0.025 mol/L) was added and the time for fibrin clotting was recorded. The activity was calculated from the heparin standard curve (clotting time *vs.* units per milligram; W. Ying *et al.*, submitted manuscript).

Flow Cytometric Assay

Cell cycle analyses were performed on EPICS-XL flow cytometer (Beckman Coulter, USA) and data were analyzed by Mcycle software. MCF-7 cells were seeded on a six-well plate and preincubated for 24 h, followed by coincubation with paclitaxel and *HD* conjugates (an equivalent paclitaxel
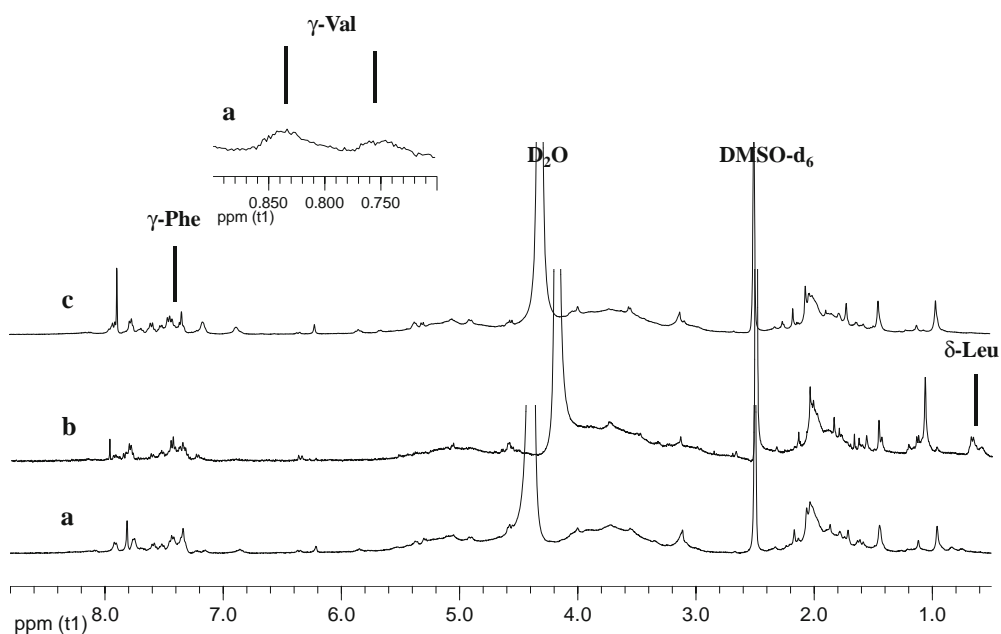

Fig. 5. ^{1}H NMR spectra of (*a*) *HD2a*, (*b*) *HD2b*, and (*c*) *HD2c* in DMSO-d$_6$ and D$_2$O (1:2).

concentration of 100 µg/mL) for 6 h. The cells were then washed three times with PBS, detached by trypsinization, spun down by centrifugation, and dispersed again in PBS for FCM analysis.

Statistical Analyses

Statistical analyses was performed to determine differences between the measures properties of group. One-way analysis of variance or independent sample t-test was determined using a statistical program (Statistical Package for the Social Sciences, Version 10.0, SPPS Inc., USA). All data were performed in triplicate and presented as a mean value with its standard deviation indicated (mean±SD).

RESULTS AND DISCUSSION

Characterization

In this study, during the preparation of *O*-acetylated heparin, carboxyl group is much more active than hydroxyl group in heparin. Therefore, mixed anhydride was formed, which facilitated linkage between drug and carrier as an active intermediate. The formation of the mixed anhydride was detected by an increase in the sulfate-to-carboxylate ratio of the *O*-acetylated heparin by conductimetric titration (W. Ying *et al.*, submitted manuscript). Besides mixed anhydride of modified heparin as intermediate, it was possible the formation of linkage between carboxyl of heparin and drug. 2'-OH of paclitaxel reacted with *O*-acetylated heparin via direct esterification reaction in the preparation of *HD1*. The previous report showed that the amino acid ester derivatives of paclitaxel were difficult to be isolated in a pure state. The instability of the 2'-glycyl ester salt was probably due to a simple inductive effect of the protonated amino group assisting in the attack of external nucleophiles on the 2'-acyl group (14). Therefore, we selected amino acids with different α-substituted group, such as isopropyl, isobutyl, and benzyl to increase the stability. Thus, we firstly synthesized 2'-amino-acyl paclitaxel, including 2'-valyl-paclitaxel, 2'-leucyl-paclitaxel, 2'-pheylalanyl-paclitaxel. In the designed *HD2* conjugates, amino acids as ideal linkers since, as bi-functional molecules, they provided a reactive carboxyl group conjugated with
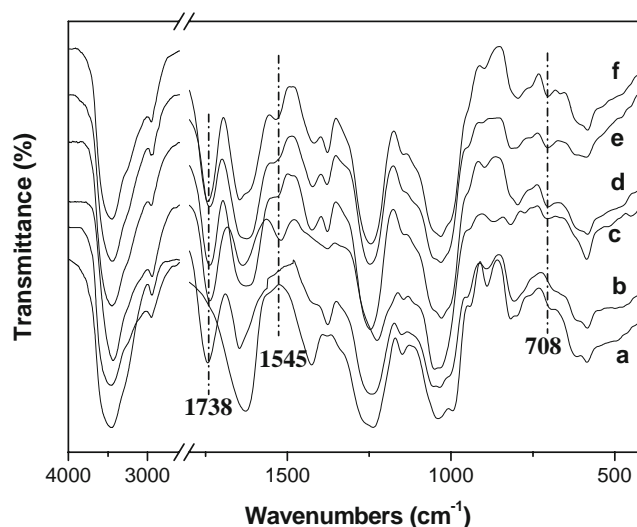

Fig. 6. FT-IR spectra of (*a*) heparin, (*b*) *O*-acetylated heparin, (*c*) *HD1*, (*d*) *HD2a*, (*e*) *HD2b*, and (*f*) *HD2c*.

paclitaxel and an amino group that could be easily linked with *O*-acetylated heparin using $HClO_4$ as catalyst.

A typical ^{1}H NMR spectra for the *HD* conjugates were shown in Figs. 3, 4, 5 and the chemical shifts ascribed to *O*-acetylated heparin, paclitaxel, and the spacer arm protons were reported in Table I. The methyl of acetyl group in chemical modified heparin was attributed to 2.1~2.2 ppm (Fig. 3). At the same time, the hydrophobicity of paclitaxel and hydrophilicity of carrier co-affected solubility of *HD1* in water. Therefore, *HD1* was observed in ^{1}H NMR spectra at co-solvent DMSO-d_6 and $CDOD_3$ (Fig. 4). However, it was unclearly detected for *HD1* at co-solvent DMSO-d_6 and D_2O. In contrast, the chemical shifts of *HD2* conjugates were clearly identified in the presence of mixing solvents DMSO-d_6 and D_2O (Fig. 5). This demonstrated that the amphiphilic nature of amino acid could improve the water-solubility of *HD2* conjugates. In Fig. 5, the proton signals of amino acid and paclitaxel could be partially observed in ^{1}H NMR spectra. Specifically, the methyl of valyl and leucyl appeared at 0.84 and 0.6 ppm in high field in spectrum a, b, respectively. Despite of the interference of the benzene ring of paclitaxel

Table I. Proton Shifts Seen for *HD* Conjugates During ^{1}H NMR Spectrum

δ (ppm)	^{1}H NMR signal descriptor	Descriptor
0.6	–CH₃, δ-Leu	**HD2b**
0.84	–CH₃, γ-Val	**HD2a**
1.25	17-CH₃	Paclitaxel, **HD**
1.27	16-CH₃	Paclitaxel, **HD**
1.58	18-CH₃	Paclitaxel, **HD**
1.76	19-CH₃	Paclitaxel, **HD**
1.9~2.0	–COCH₃,–NHCOCH₃	O-acetylated heparin
2.11	C4-OAc	Paclitaxel, **HD**
2.22	C10-OAc	Paclitaxel, **HD**
7.16	δ-Phe	**HD2c**
7.2~8.1	Benzene ring	Paclitaxel, **HD**

Table II. M_n, Polydispersity Index (PDI), Percentage Weight of Drug, and aPTT (Anticoagulant Activity) for *HD* Conjugates by GPC, UV, and aPTT Measurements, Respectively

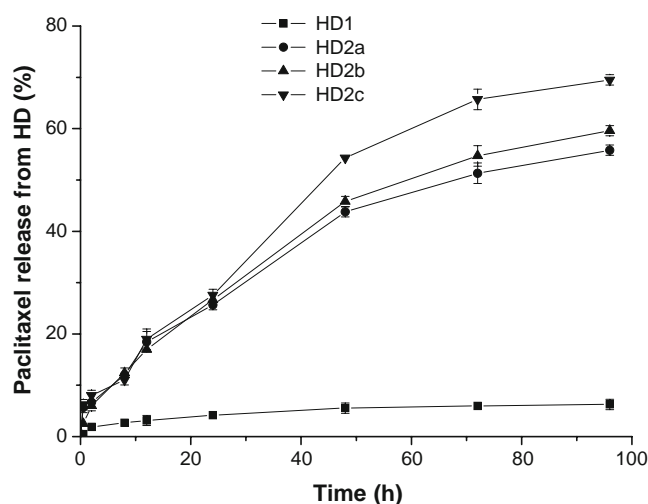| Compound	M_n	Polydispersity index (PDI)	Percentage weight of drug (*w/w* %)	APTT (U/mg)
O-acetylated heparin	12,795	1.83		127±5
HD1	18,006	2.51	25.4	25±7
HD2a	17,116	2.92	16.3	17±8
HD2b	17,213	2.78	18.4	28±4
HD2c	17,202	2.83	16.1	12±6

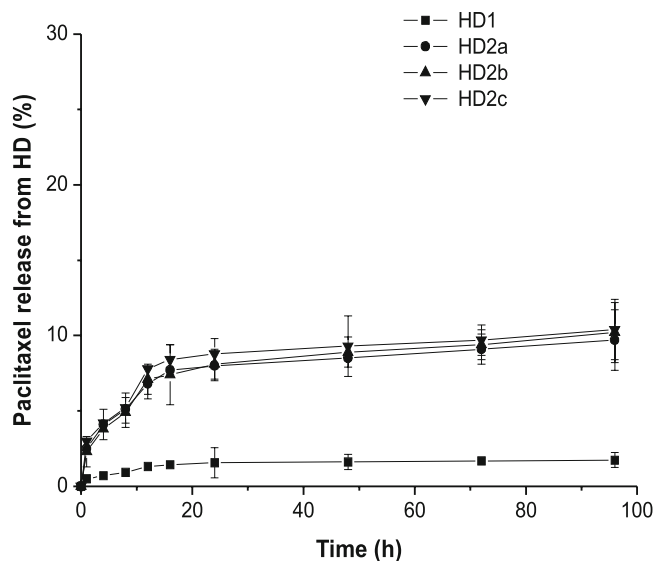**Fig. 7.** *In vitro* release profiles of paclitaxel from *HD* conjugates at pH 7.4.

Fig. 9. *In vitro* release profiles of paclitaxel from *HD* conjugates at pH 5.0.

in ^{1}H NMR, the δ-hydrogens of phenylalanyl were attributed to 7.16 ppm in low field, in spectra c.

Fig. 6 showed the IR spectra of heparin, *O*-acetylated heparin, *HD1*, and *HD2* conjugates. A new band at 1,738 cm^{-1} was assigned to the ester bond vibration in spectra b, c, d, e and f (15). After paclitaxel induced to the carrier, the band at 1,545 cm^{-1}, 708 cm^{-1} appeared in spectra c, d, e and f, which were attributed to the –C=C– stretching mode of benzene ring of paclitaxel, which indicated that paclitaxel was successfully linked to the *O*-acetylated heparin, forming *HD1* and *HD2* conjugates.

The content of drug conjugate to *O*-acetylated heparin was quantified by UV absorbance (λ=228 nm), assuming that the *HD* conjugates in water and the free drug in ethanol had the same molar extinction coefficients and that both followed Lambert Beer's law (16). The concentration of paclitaxel in

HD conjugates was estimated based on standard curve generated with known concentrations of paclitaxel in ethanol at absorption of 228 nm. The calculated weight of *HD* conjugates contained 16–25% paclitaxel (*w*/*w*; Table II). GPC measurement in aqueous phase showed the average molecular weight of *O*-acetylated heparin (M_n~12,795) with a PDI of about 1.8. However, the PDIs of Prodrugs were greatly increased to about 2.9, which might be related to the hydrophobic property of paclitaxel.

In Vitro Release of Paclitaxel from HD Conjugates

HD conjugates were disposed to chemical and enzymatic hydrolysis to test the drug release at pH 7.4 and pH 5.0, respectively. Experiments in different pH values were reported in Figs. 7, 8, 9 and 10. The *HD1* was highly stable
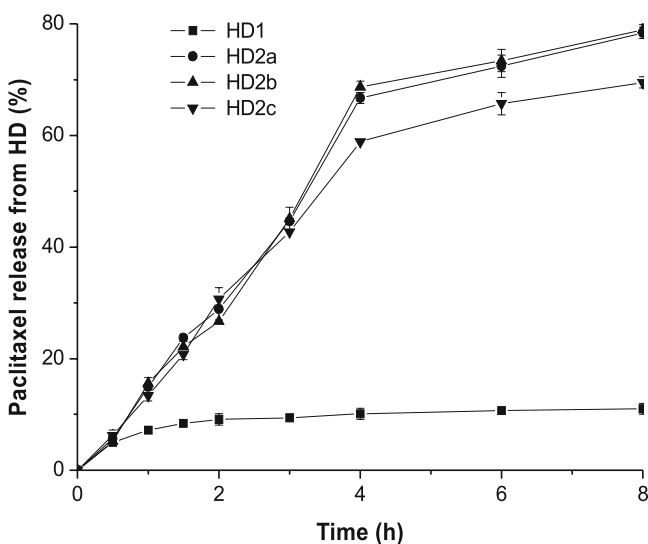

Fig. 8. *In vitro* release profiles of paclitaxel from *HD* conjugates at pH 7.4 with esterase.
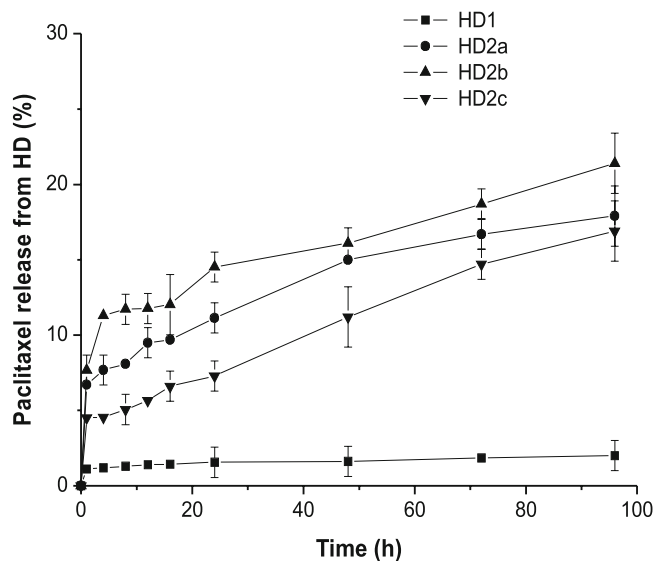

Fig. 10. *In vitro* release profiles of paclitaxel from *HD* conjugates at pH 5.0 with esterase.

under physiological and enzyme conditions. It was demonstrated that direct ester bond between carrier and drug was difficultly hydrolyzed in physiological and enzymatic condition. It was detected that over 6% of paclitaxel released from *HD2* conjugates after 2 h at pH 7.4. Meanwhile, a drug release half-life ($t_{1/2}$) of *HD2a* was similar with that of *HD2b* under physiological condition. In addition, *HD2c* showed a much faster rate ($t_{1/2}$=44 h) in physiological conditions. It was observed the hydrolysis rates of *HD* conjugates were greatly increased in pH 7.4 with esterase and hydrolysis rates of *HD2a* and *HD2b* showed faster growth trend ($t_{1/2} \approx 3.2$ h). In acidic conditions without esterase, most of paclitaxel was difficultly released from *HD2* conjugates (Fig. 9). However, under the esterase enzymatic condition at pH 5.0, about 15% of paclitaxel was liberated from *HD2a* and *HD2b* conjugates after 48 h. In contrast, *HD2c* showed higher stability under this condition compared with that of *HD2a* and *HD2b*. This might be explained by the phenylalanyl of steric hindrance, a factor that decreased the rate of release in enzymatic degradation. As a whole, *HD2* conjugates with different amino acids as linkers progressively liberated much more paclitaxel toward enzymatic condition at pH 5.0 than *HD1* conjugate. The results suggested that the molecular design of amino acids as linker facilitated the release of pacitaxel from *O*-acetylated heparin.

Anticoagulant Activity of HD Conjugates

The anticoagulant activities of *O*-acetylated heparin and *HD* conjugates were shown in Table II. The anticoagulant activities of *O*-acetylated heparin and *HD* conjugates both decreased to some extent as compared to that of heparin (150 U/mg), but those for *HD* conjugates decreased to a more large extent. This might be attributed to the introduction of paclitaxel leading to the conformational change of heparin structure, which decreased affinity to AT III and affected the underlying anticoagulant mechanism (17). Although some clinical trials suggested a beneficial effect of heparin in cancer patients, heparin therapy could be difficult to manage and of limited use due to its anticoagulant potency and potential for
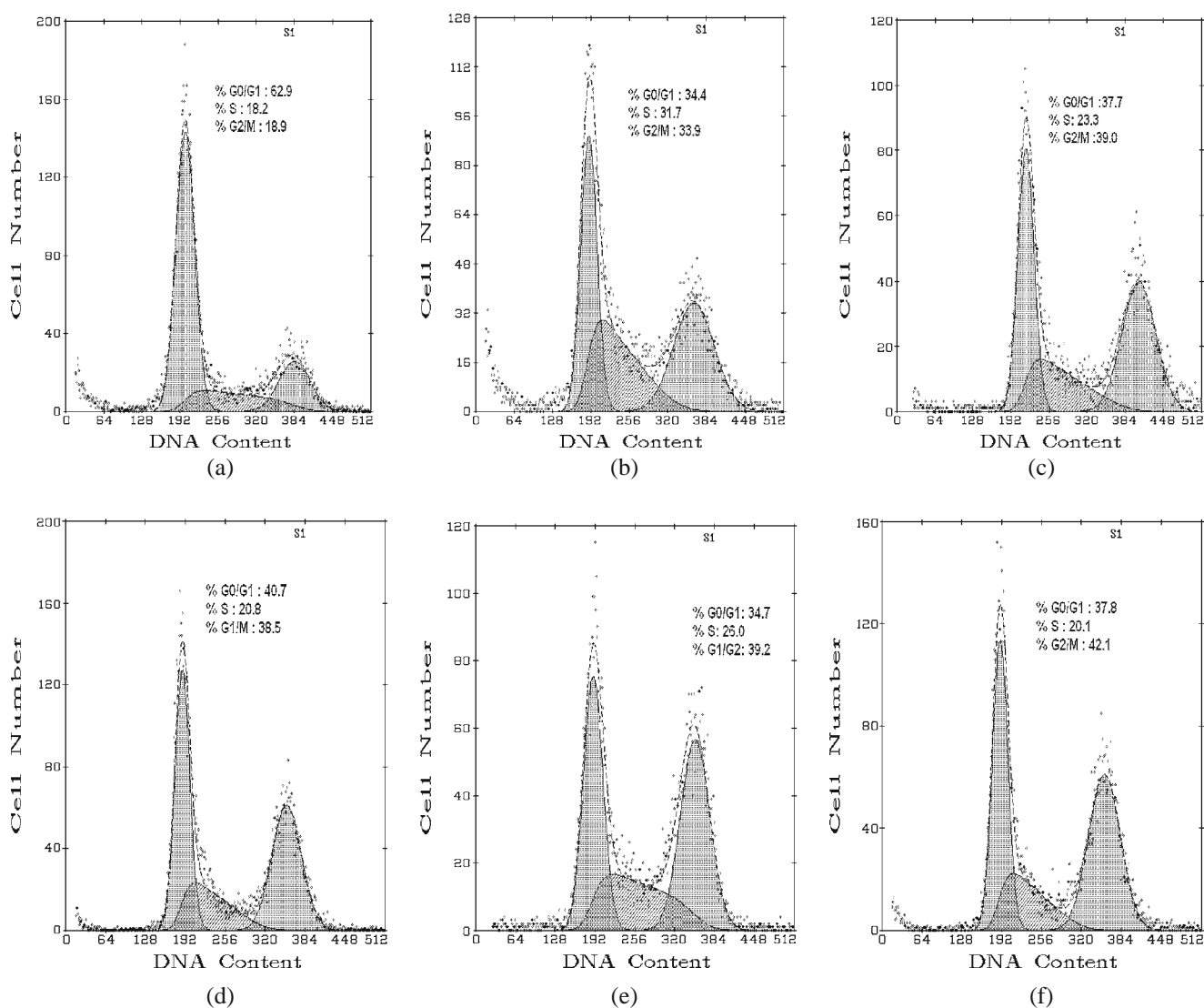
Fig. 11. Flow cytometric analysis of the cell cycle profiles of MCF-7 cell treated with paclitaxel and *HD* conjugates for 6 h. **a** Control cells, **b** paclitaxel, **c** *HD1*, **d** *HD2a*, **e** *HD2b*, and **f** *HD2c*.

inducing hemorrhagic complications. Therefore, it was needed to investigate the anticoagulant activity of *HD* conjugates. It was reported that chemical modified heparin derivatives with greatly reduced anticoagulant activity still conserved non-anticoagulant effects, including anti-adhesion, inhibition of angiogenesis, metastasis and tumor growth (18–20). As a consequence, *HD* conjugates with reduced anticoagulant activity could be safe and effective prodrugs to further investigate.

Cell Cycle Analysis of HD Conjugates

Fig. 11 showed the cell cycle profile of MCF-7 treated with paclitaxel and *HD* conjugates. It was demonstrated that since the cytotoxic activity of paclitaxel is attributed to its stabilizing effect on microtubules necessary for spindle formation and cell division, paclitaxel has been shown to cause cell cycle arrest in the G2/M phase and finally cell death through apoptotic mechanism (21,22). As compared with control cells, the sharp peak observed in the G0/G1 phase was markedly attenuated and was instead in the G2/M phase for cells treated with paclitaxel and *HD* conjugates. Although *HD1* was difficultly hydrolyzed and released from paclitaxel, we still observed cell cycle arrested in the G2/M phase. It was possibly demonstrated the whole conjugates themselves showed the same cytotoxic activity as paclitaxel. Therefore, it was expected for us to further investigate the detailed reason.

CONCLUSIONS

The heparin–paclitaxel conjugates using amino acid as the linker between carrier and paclitaxel have been investigated and compared with that of using direct ester bond. *O*-acetylated heparin with reduced anticoagulant activity has been designed to be carrier. Meanwhile, mixed anhydride groups of carrier have greatly simplified the synthesized process and improved the activity of reaction. *HD2* conjugates display better solubility in water due to the nature of amino acid. It has been demonstrated that the hydrolyzed rates for *HD2* conjugates are significantly higher than that of *HD1* under physiological condition and esterase enzymatic condition. The anticoagulant activities of *HD* conjugates sharply decrease compared with that of heparin by APTT measurement. A cell cycle analysis studied by flow cytometer has shown that *HD* conjugates can block MCF-7 cells at G2/M phase. The results indicate *HD2* conjugates offer great potential for further investigation. Importantly, *HD2* conjugates with relative lower anticoagulant property can be safe and effective prodrugs, which may avoid the high risk of excessive bleeding during clinical research.

However, anticancer property for carrier alone and in combination with drug remains to be investigated. It has been reported that the binary drug delivery system can conjugate with folic acid, which is an active recognition moiety for folate receptors highly expressed in several human tumors including ovarian and breast cancers (23,24). The ternary drug delivery system can be designed and synthesized, which composed of drug, carrier, and specific ligand-folic acid. The detailed research about the ternary drug delivery system is underway in our laboratory.

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