Pharmacokinetics and Safety Studies of Low Molecular Weight Heparin Nanoconjugates

Tianzhi Yang*, Bradley Bouchard*, Andrea Cook*, Jing Hu**, Jiukuan Hao**, Shuhua Bai*,[1]
* School of Pharmacy, Husson University, 1 College Circle, Bangor, ME 04401
** James L. Winkle College of Pharmacy, University of Cincinnati, 3225 Eden Avenue, Cincinnati, OH 45267

ABSTRACT

Venous thromboembolism (VTE), an often-fatal blood clotting disorder, which is manifested as deep venous thrombosis and pulmonary embolism, results in approximately 300,000 deaths in the United States annually. This deadly disease is often asymptomatic and may remain undiagnosed until sudden death. Low molecular weight heparins (LMWHs) have received widespread acceptance as the first choice for initial prevention and treatment of VTE via subcutaneous injection one to two times daily in the clinic. However, frequent needle-based injections and rare but serious immunogenic reactions such as heparin induced thrombocytopenia (HIT) are some important limitations that preclude the use of LMWH as a maintenance therapy for treatment of VTE disorder. Conjugation of drugs with polyethylene glycol (PEGylation) has demonstrated the wide usefulness in the improvement of therapeutic value of drugs. By increasing size of drugs and shielding them from enzymes, PEGylation leads to prolonged acting time, which allows less frequent administrations and reduce immunogenicity in many applications.

The objective of this study is to develop long-acting and safe LMWH analogs that provide significant benefits for the treatment of VTE. Our central hypothesis is that long-circulating PEGylated LMWH is a safe and viable anticoagulant therapy for the management of VTE by once-weekly subcutaneous injection.

Keywords: Low molecular weight heparin, PEGylation, nanoconjugate, anticoagulant, mitochondrial toxicity

1 INTRODUCATION

Low molecular weight heparins (LMWHs) have less molecular weight from 4,000 to 6,000 Daltons and less surface charges produced from unfractionated heparin by chemical and enzymatic polymerization. They have several advantages over unfractionated heparin including: higher bioavailability, a longer duration of action, safer, and more predictable effects than unfractionated heparin. Currently, initial subcutaneous injection of LMWH is the standard short-term prophylaxis and treatment plan for VTE (Hirsh et al., 2008). LMWH is also the drug recommended for long-term treatment for 3 to 6 months by injections for pregnant and cancer patients because it is safe and reduces complications (Falanga and Zacharski, 2005; James et al., 2005).

However, having similar side effects including injection related site reactions and thrombocytopenia with heparin, LMWHs remain important disadvantages for the long-term therapy for VTE, a chronic disease. Although the duration of action of LMWH is longer than that of unfractionated heparin, it is too short-lived for maintenance therapy. For example, the half-life (an indication of the duration of action) of enoxaparin, a widely used LMWH, is 2-3 hours (Dawes, 1990). Because their action is of short duration, LMWHs require frequent administration (once or twice daily) by painful subcutaneous injection. Needle-based drug delivery systems require the investment of extra time by skilled health care personnel and hinder administration of the drug in an outpatient setting. The net result of the frequent use of needle-sticks is poor compliance due to undesirable side effects, including pain, needle phobia, infection and hematoma at the injection site. A potential delivery approach that obviates the related hazards is to decrease the frequency of administration.

PEGylation, defined as the molecular attachment of polyethylene glycols (PEGs) to active drug molecules, has been widely used as an effective strategy to extend drug residence in the body (Jevsevar et al., 2010). PEGylation can increase solubility due to the hydrophobicity of PEG, and its increased size can decrease kidney filtration and accessibility for proteolytic enzymes. As a result, the most promising applications of PEGylation have significantly prolonged the half-life and decreased immunogenicity for many commercial therapeutic drugs, including asparaginase, adenosine deaminase, human growth hormone, interferon (IFN) alpha 2a and 2b, human granulocyte colony-stimulating factor, anti-tumor necrosis factor (TNF) antibody, uricase, aptamer, and erythropoietin (EPO). PEGylated products are administered more than once a week due to their significantly prolonged half-life in clinical settings (Cohan et al., 2011). The information regarding the long-term treatment plan for VTE clearly indicates that LMWH does not currently have optimal ease of administration and patient compliance. Actually, LMWHs are chemically suitable for PEGylation: LMWHs are polysaccharides that contain carboxylic acid groups in the glycosaminoglycan units. These actively functional groups make LMWHs suitable for chemical PEGylation. Physicochemically, LMWHs have very similar charges and molecular weights to oligonucleotide RNAs, the native form of Macugen® (Pegaptanib). In summary, the proposed product could be administered once more than a week, similar to the FDA-approved PEGylated products.

2 EXPERIMENTS

2.1 Synthesis of PEGylated LMWHs

Two PEGylated LMWHs (LP2000 and LP5000) were synthesized from linear PEGs (MW: 2,000 and 5,000) covalently reacting with LMWH using a procedure that employs standard carbodiimide chemistry modified from previous report (Jeon et al., 2008). LMWH, dissolved in a buffer solution with 2-morpholinoethanesulfonic acid and N-hydroxysuccinimide, firstly reacted with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) to activate the carboxylic acid groups of the LMWH. And then PEG-NH_2 was added into solution and reacted with activated carboxylic acid groups in LMWH to form an amide bond linker in the final PEGylated LMWH (**Figure1**). After purification by dialysis and lyophilization, the products were obtained in a powder form.

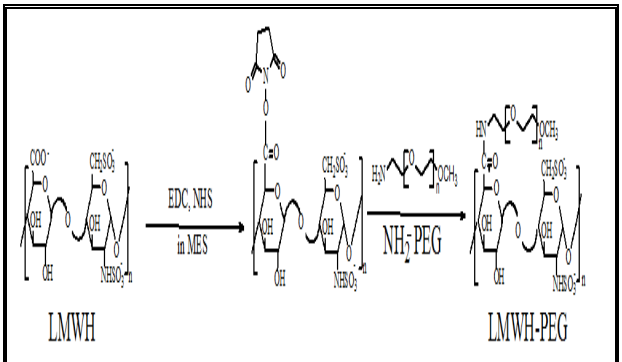

Figure 1. A scheme for the synthesis of PEGylated LMWHs.

Chemical structures of products were confirmed according to our previous studies (Bai et al., 2007; Bai and Ahsan, 2009). Attenuated Total Reflectance Fourier transform infrared (FTIR) spectra were recorded on a Nicolet Nexus 470 spectrometer (Thermo Nicolet Corp, Madison, WI) using the Smart Miracle ATR accessory between 4000 and 700 cm^{-1} at a resolution of 1 cm^{-1}. ^1H nuclear magnetic resonance (NMR) was used to analyze the couple ratio of PEG to LMWH. ^1H NMR spectra were recorded on a Varian Mercury Plus 300 MHz spectrometer (Varian Inc., Fort Collins, CO).

2.2 Characterization of PEGylated LMWHs

The size and zeta potential of PEGylated LMWHs were measured by a Delta Nano sizing system (Beckman Corp., Brea, CA) to determine if there is any decrease or increase in the size and charge after PEGylation. Activities of PEGylated LMWHs were determined by measuring anti-factor Xa activity using a colorimetric assay kit as described before (Yang et al., 2006; Bai et al., 2009). In this bioassay, LMWHs form a complex with antithrombin and the mixture was incubated with an excess of factor Xa. An amount of factor Xa was neutralized by the heparin-antithrombin complex in proportion to the available amount of heparin. The remaining amount of factor Xa hydrolyzed the chromogenic peptide substrate thus liberating the chromophoric group, paranitroaniline. After stopping the reaction with acetic acid, the intensity of the color was measured spectrophotometrically at 405 nm.

2.3 *In vivo* Pharmacokinetic Studies

Experiments were carried out using Sprague-Dawley rats. Synthesized PEGylated LMWHs in normal saline were administered subcutaneously (50 U/kg) to anesthetized rats. Blood samples were collected from 0.1 to 24 hours. Anti-factor Xa activity, which reflects LMWH levels in plasma, was analyzed according to our previous studies (Bai et al., 2009; Bai and Ahsan, 2009).

2.4 Cytotoxic Studies

Experiments were conducted to address the nonspecific cell toxicity of PEGylated LMWHs measured as cell viability using the MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazol-ium bromide) assay as previously described (Bai et al., 2007). Products were tested for their cytotoxic effect in human hepatic HepG2 cells. Cells were seeded in 96-well plate and incubated with product or control samples. And then the MTT solution was added to each well and incubated. Next, the solution in each well was removed and acidified isopropyl alcohol was added to dissolve purple crystalline. Finally, the plates were measured on a Synergy 4 microtiter-plate reader (Biotek, Winooski, VT) at 570 nm. Cell viability was expressed as the percentage of MTT released by cells exposed to all the treatments.

2.5 Analysis of Mitochondrial Toxicity

Basal oxygen consumption and glycolysis rates were used to assess mitochondrial dysfunction in hepatic HepG2 cells as previous reported (Dykens et al., 2008). Briefly, cells were seeded in XF 24-well cell culture microplates to allow media temperature and pH to reach equilibrium before the first rate measurement. Prior to the rate measurements, the assay media were mixed in each well to allow the oxygen partial pressure to reach equilibrium in the XF24 Analyzer (Seahorse biosciences, North Billerica, MA). And the test samples as well as control group were injected into plate at the time points indicated. Different enzyme levels oxygen consumption rate (OCR) - a measure of mitochondrial respiration, as well as the extracellular acidification rate (ECAR) - a measure of glycolysis, were measured. The results were expressed as a percentage of OCR or ECAR change over baseline.

3 RESULTS AND DISCUSSION

The chemical structures of the PEGylated LMWHs (LP2000 and LP5000) were confirmed by fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectra. FTIR spectra were used to confirm the amide bond linker: a strong absorption peak representing the stretching vibration of the carbonyl group (Vc=o, 1668 cm^{-1}) in the linkers is observed, as shown in **Figure 2**. ^{1}H NMR was used to analyze the protons of PEG attached to LMWH. The protons of PEG and LMWH were observed in the spectra of the products, as shown in **Figure 3**. These spectra indicated that PEGylated LMWHs were successfully synthesized.

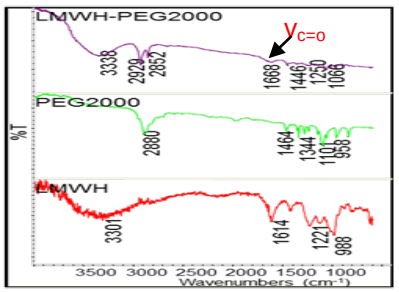

Figure 2. Stacked FTIR spectra of LMWH, PEG2000, and LP 2000.

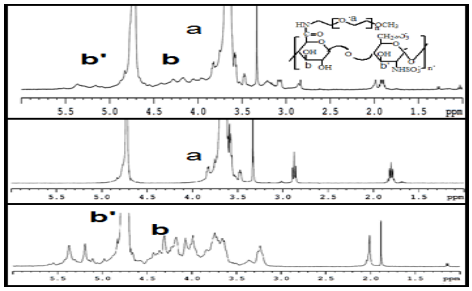

Figure 3. ^{1}H-NMR spectra of LMWH, PEG2000, and LP 2000.

The synthesized products were characterized using a Beckman Delta nanosizing system. Nanosize (230 and 320 nm) and less negative zeta potential (-20 and -11 mV) were observed (**Table 1**). The products exhibited potential anti-factor Xa anticoagulant activities as determined using a colorimetric assay (Yang et al., 2006; Bai et al., 2009); in particular, modified LMWHs maintained anticoagulant effects for longer time (i.e., they exhibited a longer stability half-life) in simulated physiological fluid including heparinase at 37 °C (**Table 1**). The results indicated that the new LMWH analogs had long-acting anticoagulant activity *in vitro*.

Table 1. *In Vitro* Properties of PEGylated LMWHs. Data represent the means ± SD, n=3-5 (* indicated a significant difference, p<0.05).

Name	Size (nm)	Zeta potential (mV)	Activity U/nmol	Half-life (hr)
LMWH	0	-34±1	204	3±1
LP2000	230±6*	-20±2*	198±4	25±2*
LP5000	320±16*	-11±3*	197±11	87±22*

In pharmacokinetic studies, the increases in $AUC_{0\rightarrow\infty}$ and half-life ($t_{1/2}$) clearly suggested that the PEGylated LMWHs had prolonged anticoagulant activities after administration. In particular, PEG5000 modified LMWH demonstrated the half-life of 17 hours and possessed more than 0.15 U/ml of anti-factor Xa activity after 24 hours, which indicated that this drug could be considered a feasible candidate for the long-term management of VTE when administered once weekly (**Figure 4 and Table 2**).

Table 2. Pharmacokinetic parameters of PEGylated LMWHs. Data represent the mean ± SD, n=3-5 (* indicated a significant difference, p<0.05).

Name	C_{max} (U/ml)	T_{max} (hr)	AUC (U·hr/ml)	$t_{1/2}$ (hr)
LMWH	0.27±0.03	1.7±0.6	2.1±0.9	4.1±1.0
LP2000	0.28±0.03	2.0±0	8.4±1.0*	10.9±3.0*
LP5000	0.31±0.04	0.9±0.7	14.7±3.4*	17.5±7.2*

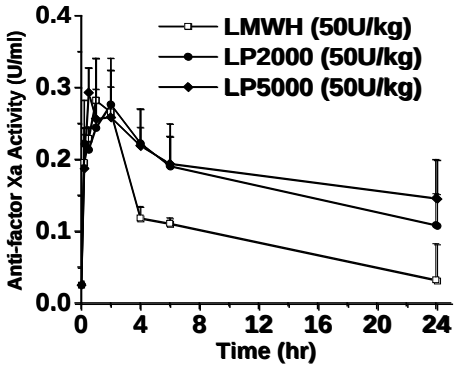

Figure 4. Plasma concentration versus time for PEGylated LMWHs. The data represent the mean ± SD, n=3-5.

MTT assay was used to examine the cytotoxic effects of PEGylated LMWHs on HepG2 cells. A positive control using 1 mg/mL of SDS produced a cell viability of 47.6±5.2%. In contrast, there were high levels of viability for the cells after incubation with the PEGylated LMWHs. All three LWMHs had cell viabilities greater than 95% and had no significant differences compared to the control medium treatment ($p>0.05$) (**Figure 5**). From the data above, the PEGylated LMWHs did not demonstrate any cytotoxicity toward hepatic HepG2 cells compared to native LMWH.

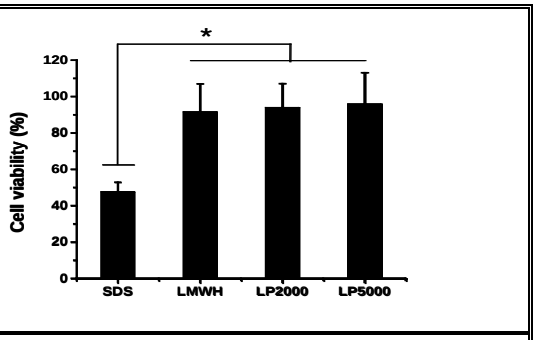

Figure 5. Cell viabilities of HepG2 cells after treatments (* indicated significant difference, p<0.05).

In vitro mitochondrial toxicity testing methods have been used to confirm the lack of certain toxic properties in the early stages of the development of potentially useful anticoagulant therapeutic drugs (Dykens and Will, 2007). Activities of various enzymes indicated by the oxygen consumption rate (OCR, which measures mitochondrial respiration) and the extracellular acidification rate (ECAR, which measures glycolysis), were measured (**Figures 6**). No any mitochondrial toxicity toward hepatic HepG2 cells caused by PEGylated LMWHs was observed.

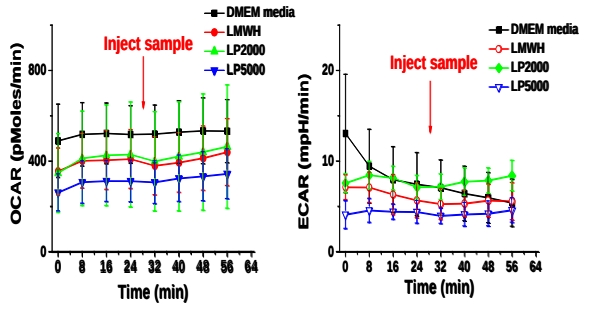

Figure 6. Metabolic profiling of oxygen consumption (OCR) and acidification rate (ECAR). Data were collected simultaneously and products were injected to 10 mg/ml final concentrations, respectively.

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[1] Contact author, School of Pharmacy, Husson University, 1 Collge Circle, Bangor, ME
Phone: 207-9921949; Fax: 207-9921945
E-mail: BaiS@husson.edu