European Journal of Pharmaceutics and Biopharmaceutics 80 (2012) 323–331

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Loading 3-deazaneplanocin A into pegylated unilamellar liposomes by forming transient phenylboronic acid–drug complex and its pharmacokinetic features in Sprague–Dawley rats

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ARTICLE INFO

Article history:
Received 28 June 2011
Accepted in revised form 22 October 2011
Available online 28 October 2011

Keywords:
3-Deazaneplanocin A
Phenylboronic acid
Pegylated liposomes
Pharmacokinetics
Nucleoside analogs
Epigenetic anticancer agent

ABSTRACT

3-Deazaneplanocin A (DZNep) is an attractive epigenetic anticancer agent through the inhibition of the cellular enhancer of zeste homolog 2 (EZH2) protein. The purpose of this study was to improve the pharmacokinetic characteristics of DZNep *in vivo* through developing a unilamellar pegylated liposomal formulation encapsulating DZNep (L-DZNep). A remote-loading method in the presence of phenylboronic acid (R-w-PBA) was developed to stably encapsulating DZNep inside liposomes (encapsulation efficiency = 50.7% at molar ratio of 1:10 of drug to lipids) through forming a transient PBA–DZNep complex. The pharmacokinetics of L-DZNep was investigated in Sprague–Dawley rats. In comparison with free drug, encapsulation of the DZNep in pegylated liposomes resulted in 99.3% reduction of the plasma clearance, whereas it increased the elimination half-life from 1.1 h to 8.0 h and the area under the plasma concentration curve by 138-fold. These findings demonstrate a novel approach (R-w-PBA method) through the development of L-DZNep, which may be extensively applied for the encapsulation of hydrophilic nucleoside analogs containing vicinal hydroxyl groups and protonable amino in the pegylated liposomes. Additionally, the pegylated liposomes could effectively prolong the retention of DZNep in the systemic circulation and therefore is highly likely to increase the DZNep's tumor localization.

1. Introduction

3-Deazaneplanocin A (DZNep), a cyclopentenyl analog of 3-deazaadenosine, recently appears to be a unique chromatin remodeling (epigenetic) compound that can deplete the cellular enhancer of zeste homolog 2 (EZH2) protein, which is abnormally overexpressed in varied metastatic cancers [1,2] and therefore inhibits the associated histone methylation [3–5]. Consequently, it can effectively reverse EZH2-mediated gene silencing and induce cancer cell death but not in corresponding normal cells.

The preliminary preclinical pharmacokinetic studies indicated that, following intravenous administration to Sprague–Dawley rats, DZNep had an undesirable pharmacokinetic profile with a short elimination half-life (1.1 h). DZNep also showed a low protein binding in plasma (18.5%) and a low partitioning to erythrocyte (0.78), but had extensive tissue distribution and predominant renal excretion (80.3%). Besides, DZNep appeared to induce the nephrotoxicity and renal atrophy in a dose-dependent manner in previous acute toxicity study in rats (unpublished).

To overcome these shortcomings of DZNep in pharmacokinetic and toxic properties, the ideal scenario would be to sequester DZNep in a package, which has minimal interactions with healthy cells, and to release drug at an appropriate time from the sequestering carrier at the tumor site. Generally, several drug delivery systems, namely liposomes, microparticles, nanoparticles, polymeric micelles, dendrimers, hydrogels, polyplex nanogels, and cyclodextrin inclusion complexes [6], have been introduced to overcome some limitations of drug *in vivo*. Liposomal formulation,

Abbreviations: ANOVA, one-way analysis of variance; $AUC_{0-\infty}$, area under the concentration–time curve from time zero to infinity; BW, body weight; cis diol, vicinal hydroxyl groups; CL, clearance; C_{max}, maximum plasma concentration; Cr, creatinine; DZNep, 3-deazaneplanocin A; EZH2, enhancer of zeste homolog 2; IS, internal standard; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; L-DZNep, pegylated liposomal formulation encapsulating DZNep; LLOQ, lower limit of quantitation; $Log K_{ow}$, the logarithm of the water–octanol partition coefficient; MAC, membrane attack complex; MLV, multilamellar; MPS, mononuclear phagocyte system; MRT, mean residence time; NUS, National University of Singapore; PBA, phenylboronic acid; PBS, phosphate-buffered saline; PEG, polyethylene glycol polymer chains; PK, pharmacokinetics; r^2, coefficient of determination; REV, reverse-phase evaporation; RP, reversed-phase; RSD, relative standard deviation; R-w-PBA, remote-loading method in the presence of phenylboronic acid; R-wo-PBA, remote-loading method in the absence of phenylboronic acid; SD, standard deviation; SUV, small unilamellar liposomes; TEM, transmission electron microscopy; UV, ultraviolet; V_{ss}, apparent volume of distribution at steady state.

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doi:10.1016/j.ejpb.2011.10.014

in particular, offers the simplest possibility to modulate the biopharmaceutical properties of drug and thus would be employed in this study. The stealth liposomes with polyethylene glycol polymer chains (PEG) coating have been proven to possess a longer circulatory life in the drug delivery [7]. Moreover, the small unilamellar liposomes (SUV) with size level around 100 nm are capable of efficient extravasation through fenestrated endothelium of tumor blood vessel walls [8].

Generally, it is very difficult using presently available technologies to stably encapsulate polar and water-soluble nucleoside analogs into conventional liposomes as these molecules may diffuse rapidly through liposome bilayers. Shortly after their preparation, the nucleoside analogs rapidly diffuse out of the liposomes, which would limit the shelf life and the clinical use of conventional liposomes [6]. Therefore, the effective methods for stably encapsulating nucleoside analogs require the interaction between nucleoside analogs and inner water phase. Due to the presence of a readily protonable amino group in the DZNep (p*K*a = 6.22, Sparc on-line calculator, http://ibmlc2.chem.uga.edu/sparc/smiles/smiles.cfm) at a weakly acidic condition, a remote-loading technique is likely to be suitable for the preparation of DZNep pegylated liposome (L-DZNep). The principle of this technique is that the free alkaline drug would diffuse inside the liposome following pH gradient (4.0–7.0), where protonation of the drug occurs and in turn results in its accumulation inside the liposomes [9,10]. On the other hand, boric acids, such as phenylboronic acid (PBA), were found to facilitate the transport of ribonucleosides or nucleoside analogs containing vicinal hydroxyl groups (cis diol) through lipid bilayers, which are involved in a transient formation of a lipophilic, trigonal boronate ester [11,12]. Since DZNep also possesses a cis diol in its cyclopentenyl moiety as well as a protonable amino group, it was thus hypothesized that the encapsulation of DZNep in the pegylated unilamellar liposomes was feasible using the remote-loading technique with the aid of PBA and that this formulation was capable of prolonging the retention of DZNep in the systemic circulation of SD rats as compared to the free drug. Thus, the main purpose of this study was to improve the pharmacokinetic characteristics of DZNep *in vivo* through developing a pegylated unilamellar liposomal formulation.

2. Materials and methods

2.1. Chemicals

3-Deazaneplanocin A (DZNep, purity >99%) was purchased from OKeanos Tech. Co. Ltd. (Beijing, China). Tubercidin (purity >99%) was from Sigma (St. Louis, MO, USA). Phenylboronic acid (PBA, purity >95%), ribavirin, and ammonium sulfate were also from Sigma. Lipids such as cholesterol, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glyco)-2000] (Ammonium salt) (PEG2000-DSPE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). HPLC-grade acetonitrile was obtained from TEDIA (Fairfield, USA). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). All other chemicals and solvents used were obtained from standard vendors and were of the high quality available.

2.2. HPLC–UV assay for the determination of DZNep and an additive, PBA in vitro

Shimadzu HPLC system (Shimadzu, Kyoto, Japan) used was equipped with an SCL-10Avp system controller, LC 10 ATvp pump, DGU-14A degasser, SIL-10ADvp autosampler, and SPD-M10Avp UV/Vis photodiode array (PDA) detector. Chromatographic separation was conducted on a phenyl reversed-phase column (XTerra™ Phenyl, 4.6 × 150 mm, particle size of 5 μm). The mobile phase consisted of 10 mM phosphoric acid and 20 mM disodium phosphate in water. The flow rate was 1.5 mL/min and the temperature was ambient. The eluent of DZNep, PBA, and ribavirin (IS) was monitored at a wavelength of 215 nm. Chromatographic peaks were electronically integrated and recorded using Class VP software (ver. 6.12, Shimadzu, Japan).

DZNep liposomes (L-DZNep) were diluted five times with PBS buffer (pH 7.4); 100 μL of diluted L-DZNep was spiked with 10 μL of 100 μg/mL of ribavirin as the internal standard. Then, DZNep and PBA encapsulated in the diluted L-DZNep were released by lysing liposomes with 90 μL of 2% Triton X-100 solution. Then, each sample was incubated in a water bath at 60 °C until the cloud point of the detergent was observed. In the same way, blank liposome lysis was obtained through lysing blank liposomes. Each prepared sample (200 μL) was pipetted into an autosampler vial, and 20 μL was injected onto column for analysis.

The method validation for assaying DZNep and PBA in liposomes was as follows. The selectivity of the method was evaluated by comparing the chromatograms of five blank samples with the corresponding spiked samples at the lower limit of quantitation (LLOQ) level. Calibration standards at final concentrations of 0.5, 1, 5, 10, 20, 50, and 100 μg/mL of DZNep and of 1, 5, 10, 50, 100, 500, and 1000 μg/mL of PBA were prepared. All standards were spiked with final concentration of 5 μg/mL IS. The calibration curves were fitted by a linear least-squares regression method through the measurement of the peak-area ratio of DZNep to IS versus the corresponding known spiked concentrations. The coefficient of determination (r^2) greater than 0.99 was set as the acceptable linearity for calibration curves. The accuracy and precision at the LLOQ for each sample were required within 20% of the nominal concentration and less than 20% RSD (n = 5), respectively.

2.3. Two physicochemical properties of DZNep

2.3.1. Measurement of the logarithm of the water–octanol partition coefficient of DZNep (Log K_{ow})

The Log K_{ow} of DZNep in pH 7.4 (unionized basic compound) was measured using the shake flask method, in accordance with the Guidelines for Testing of Chemicals by the Organisation for Economic Cooperation and Development (OECD) [13]. Briefly, water-saturated 1-octanol solution and 1-octanol-saturated PBS buffer (pH 7.4, 1stBase) were prepared, and DZNep was then dissolved in the 1-octanol-saturated PBS buffer at the final concentration of 5, 10, and 50 μg/mL; 5 mL of the above DZNep solution was added to 5 mL of the water-saturated 1-octanol solution in a glass flask. The heterogeneous mixture was stirred (180 rpm) at room temperature overnight and then centrifuged for 10 min at 4500 g using a Sigma203 Centrifuge (Deisenhofen, Germany). Following separation, 100 μL of aqueous sample and of 1-octanol sample were collected. 1-Octanol was evaporated using a stream of nitrogen at 45 °C overnight. The residue was reconstituted in 100 μL of PBS buffer (pH 7.4). Concentrations of DZNep in octanol and aqueous phase were analyzed using the HPLC–UV method. Log K_{ow} was calculated by taking the logarithm of the ratio of the concentration of DZNep in 1-octanol to the corresponding concentration in PBS buffer.

2.3.2. Thermostability of DZNep in acidic and neutral aqueous buffers

Due to the high temperature (60 °C) and pH gradient (4.0–7.0) involved in the remote-loading method for the preparation of liposome containing DZNep, a thermostability test was performed through incubating DZNep in acidic and neutral aqueous solution at 60 °C; 50 μg/mL of DZNep in citrate buffer (pH 4.0) and 5% glucose (pH 7.0) were prepared. Aliquots (0.5 mL) of DZNep samples

were sealed in 1.5-mL Eppendorf tubes and incubated in the water bath at 60 °C for 2 h. Thereafter, DZNep concentration in each sample was measured using the HPLC–UV method. Freshly prepared DZNep samples (50 μg/mL) with different pH value served as control. The fraction of remaining DZNep was determined by dividing DZNep concentration in the incubated sample by that in the control sample.

2.4. Preparation of DZNep-loaded liposomes (L-DZNep)

L-DZNep was obtained using three different preparation methods: the reversed-phase evaporation (REV), remote-loading in the absence of PBA (R-wo-PBA), and remote-loading in the presence of PBA (R-w-PBA). Based on the optimum encapsulation efficiency of DZNep in different formulation of L-DZNep, the molar input ratio of DSPC, cholesterol, and PEG2000-DSPE was 60:35:5, while that of DZNep and lipids was 1:10 (see Supplementary Fig. 1). The liposomes were prepared by sequential extrusion at 60 °C through Nuclepore® Polycarbonate membranes (Whatman®, USA) with pore sizes of 0.2 μm and 0.1 μm using a mini-extruder set (Avanti Polar Lipids, Inc., USA).

2.4.1. Reversed-phase evaporation method (REV)

As REV method is an effective way of encapsulating hydrophilic drugs [14], it was initially selected for optimization. L-DZNep was prepared using the reverse-phase evaporation (REV) method as described previously [15] with minor modifications. In brief, lipid materials were first dissolved in 15 mL of a solvent mixture containing chloroform and isopropyl ether (1:2, v/v) and sonicated in a bath-type sonicator (Ultrasonicator FB 15057, Fisher Scientific) at 45 °C for 1 min under N_2 to ensure homogeneous mixing. The water phase containing 1 mg/mL of DZNep in 5% glucose solution (equal to 1/3 volume of solvent mixture) was added to the organic solvent phase, and the mixture was sonicated for 4 min. The organic solvent was removed under vacuum on a rotary evaporator at 60 °C for about 1 h, leaving white and gel-like multilamellar (MLV) liposomes. Size reduction of liposomes was carried out by sonication followed by sequential extrusion at 60 °C. As such, the DZNep-loaded small unilamellar (SUV) liposomes were obtained. The final lipid concentration was approximately 40 mM, while the DZNep concentration was around 1 mg/kg.

The coarse L-DZNep was then subject to five consecutive dialysis cycles against 50 mL of 5% glucose solution to remove unencapsulated DZNep, each for 1 h. L-DZNep prepared by this REV method was stored at 4 °C.

2.4.2. Remote-loading without the presence of PBA (R-wo-PBA)

Preparation of blank SUV liposomes and ammonium sulfate gradient formation: blank SUV liposomes were prepared using the reverse-phase evaporation (REV) method, and ammonium sulfate gradient was generated as described previously [9] with some modifications. Of notice, the water phase consisted of 250 mM ammonium sulfate solution instead of 1 mg/mL of DZNep in 5% glucose and other procedures were same as the above-mentioned REV method until the extrusion step. SUV blank liposomes containing 250 mM ammonium sulfate solution were then subject to five consecutive dialysis cycles against 50 mL of 5% glucose solution, each for 8 h. This dialysis removed most of the unencapsulated ammonium sulfate and established a transmembrane ammonium sulfate gradient. The prepared liposomal mixtures were stored at 4 °C for at most 1 day.

Drug uptake in liposomes: 10 mg/mL of DZNep in 5% glucose solution was incubated with blank liposomes with a DZNep-to-lipid molar ratio of 1:10 at 60 °C for 2 h. The final DZNep concentration in the mixture was approximately 1 mg/mL. The mixtures were allowed to cool to room temperature (25 °C), and any unencapsulated DZNep was removed through five consecutive dialysis cycles with 50 mL of 5% glucose solution using dialysis membrane tubing (Spectra/Por Biotech, USA), each for 1 h.

2.4.3. Remote-loading with the presence of PBA (R-w-PBA)

For R-w-PBA, PBA was mixed and incubated with DZNep in 5% glucose solution (pH = 7.0 ± 0.1) in the PBA-to-DZNep molar input ratios of 1:1, 4:1, 6:1, 8:1, and 10:1 at 60 °C for 5 min before this mixture was incubated with blank liposomes in the DZNep-to-lipid molar ration of 1:10. The final DZNep concentration was around 1 mg/mL. Then, this mixture was incubated with blank liposomes at 60 °C for 2 h. The subsequent steps were similar to the preparation of R-wo-PBA as described above.

2.5. Characteristics of L-DZNep

2.5.1. Encapsulation efficiency of DZNep or PBA in liposomes

The encapsulation efficiency of DZNep or PBA into liposomes was determined by dialysis. Briefly, L-DZNep samples were collected equally before and after the unencapsulated drugs were removed by dialysis. One treated by dialysis was denoted as dialysis sample. The other was denoted as initial sample. Two sets of samples were diluted to the same volume using 5% glucose solution. Both were subject to treatment with 2% Triton X-100 solution as described previously to release encapsulated drugs. DZNep or PBA concentration was quantified using the HPLC–UV method. Thus, encapsulation efficiency was determined using the following equation,

$$\text{Encapsulation efficiency} = \frac{[\text{Drug}]_{\text{dialysis}}}{[\text{Drug}]_{\text{initial}}} \times 100\%$$

where $[\text{Drug}]_{\text{initial}}$ refers to the DZNep or PBA content in the initial sample, while $[\text{Drug}]_{\text{dialysis}}$ to the encapsulated DZNep or PBA in the dialysis sample.

2.5.2. Zeta potential and size of L-DZNep

The dispersion of L-DZNep (50 μL) was diluted with 2 mL of 5% glucose solution and phosphate-buffered solution (PBS, invitrogen, USA). The zeta potential, size, and polydispersity of L-DZNep were measured using dynamic light scattering (Zetasizer 3000HS, Malvern Instruments, UK).

2.5.3. Microscopic observation of L-DZNep

The L-DZNep was observed using a transmission electron microscopy (TEM). A drop of the sample dispersion was placed on a 100 mesh copper grid, and then the excess dispersion was removed with a piece of filter paper. A drop of 2% phosphotungstic acid solution (pH 7.4) was added to the grid and then dried for 1 h in a desiccator. The morphology of the L-DZNep was observed by TEM (JEM-3010, JEOL, Japan).

2.5.4. In vitro release of encapsulated DZNep from L-DZNep

The *in vitro* release of encapsulated DZNep from L-DZNep was performed as described by Meng et al. [16]. Briefly, the release of DZNep from L-DZNep was performed against 5% glucose solution using the dialysis membrane tubing (Spectra/Por Biotech, USA) with molecular weight cutoff of 12 kDa. Aliquots of 1 mL of L-DZNep diluted with 5% glucose solution or plasma at 1:1 ratio were placed in dialysis tubing, which were immersed in 100 mL of 5% glucose and then incubated at 37 °C with constant stirring (500 rpm) using magnetic heat stirrer. At 0.5, 1, 2, 3, 4, 5, 6, 9, 12, 24, 36, and 48 h, 100 μL of the exterior medium was aliquoted out and replaced with 100 μL fresh medium. As the recipient samples contained substances from rat plasma, the concentration of

DZNep in such a medium was analyzed using a LC–MS/MS method as described previously [17]. The cumulative release percentages of DZNep were calculated as the DZNep content in the exterior medium at each time point divided by the DZNep initially added in the dialysis tubing.

2.6. Pharmacokinetics studies of L-DZNep

2.6.1. Animals

The female Sprague–Dawley rats were provided by Laboratory Animal Center (National University of Singapore, Singapore) and housed in a temperature-controlled room (25 °C) with a 12-h light–dark cycle. The research protocol was approved in advance by the Animal Ethics Committee of National University of Singapore. Sterile polyethylene cannula (PE50, I.D. 0.58 mm, Becton Dickinson) was implanted in the jugular vein of rats under ketamine/xylazine anesthesia as described by Thrivikraman et al. [18], and animals were allowed to recover from surgery for 1 to 2 days.

2.6.2. Pharmacokinetic experiments

Twelve female SD rats were randomly divided into two groups (each group consisting of six rats). One group was given a single intravenous dose of DZNep solution at 1 mg/kg, while the other a single dose of L-DZNep, also at 1 mg/kg, via the vein tail (both preparations consisted of 0.5 mg/mL DZNep in 5% glucose solution). At 0, 0.016, 0.083, 0.17, 0.5, 1, 3, 6, 9, 12, 24, and 48 h after dosing, 0.2 mL of blood samples was collected via the jugular vein cannula in heparinized polypropylene tubes. The plasma was separated by centrifuging the blood samples for 10 min at 2000 g and stored at −20 °C until analysis using the LC–MS/MS method with a modified sample pretreatment. Briefly, 10 μL 10% Triton X-100 was added to aliquots of 100 μL of plasma samples. The mixture was vortexed for 1 min to release the remaining of encapsulated DZNep in L-DZNep. The subsequent procedure was then subject to the same assay method for rat plasma samples containing free DZNep [17]. Briefly, the 100 μL of tubercidin (IS) working solution (100 ng/mL) was added. Then, the plasma samples were mixed with 1 mL of 0.2 M ammonium acetate buffer (pH 9.0). The Bond Elute PBA-SPE cartridges (Varian, Palo Alto, CA) were preconditioned by consecutive washing with 1 mL methanol, 1 mL 0.1 M formic acid, and 1 mL 0.2 M ammonium acetate buffer (pH 9.0). The mixtures were loaded into the column. Then, the columns were washed thrice with 1 mL 0.2 M ammonium acetate buffer (pH 9.0). Samples were eluted with 1.5 mL 0.1 M formic acid and freeze-dried overnight using a lyophilizer (Labconco, Kansas City, MO). Following this, the freeze-dried residues were reconstituted in 100 μL mobile phase, and 10 μL was injected onto column for analysis. The chromatographic separations of DZNep were performed on a hydrophilic interaction chromatography column (2.1 × 50 mm, Zorbax HILIC plus, Agilent) with a Silica Security Guard Cartridge (3.0 × 4 mm, Phenomenex). The mobile phase was made up of solvents A (H_2O and 0.1% formic acid) and B (acetonitrile and 0.1% formic acid) at a flow rate of 0.3 mL/min. DZNep and tubercidin (IS) were quantified using the multiple reaction monitoring (MRM) mode with the following transitions: *m/z* 263.3/135.1 for DZNep and *m/z* 267.2/135.0 for tubercidin (IS), respectively.

2.6.3. Pharmacokinetics analysis

The plasma pharmacokinetic parameters describing the DZNep plasma concentration curve were assessed using model-independent, non-compartmental analysis with WinNonLin 5.0.1 (Pharsight, Mountain View, CA). The pharmacokinetic parameters describing the DZNep plasma concentration–time curve were estimated for each rat and expressed as the means ± standard deviation (SD). The initial concentration (C_0) after intravenous injection was determined by extrapolating the curve to $t = 0$ obtained through linear regression on the logarithmic transformation using the first several data points. The terminal plasma half-life ($t_{1/2,\ \lambda z}$) was calculated as $0.693/\lambda_z$. The volume of distribution during the terminal phase (V_z) was estimated as CL/λ_z. The area under the plasma concentration–time curve (AUC_{0-t}) and the area under the first moment curve ($AUMC_{0-t}$) from time zero to the last measurable time (t_{last}) were calculated using the log-linear trapezoidal rule. AUC from time zero extrapolated to infinity ($AUC_{0-\infty}$) was calculated as follows: $AUC_{0-\infty} = AUC_{0-t\text{last}} + C_{last}/\lambda_z$, where C_{last} is the last measured plasma concentration and λ_z is the elimination rate constant calculated from the slope of the terminal log-linear phase of the plasma concentration–time curve. The area under the first moment curve from time zero to infinity ($AUMC_{0-\infty}$) was calculated as the sum of $AUMC_{0-t}$ and the extrapolated area, which was estimated as $(t_{last} \times C_{last}/\lambda_z + C_{last}/\lambda_z^2)$. The mean residence time (MRT) was determined as $AUMC_{0-\infty}/AUC_{0-\infty}$. The plasma clearance for intravenous dose (CL) was calculated as dose/$AUC_{0-\infty}$. The volume of distribution at steady state (V_{ss}) was estimated as CL × MRT.

2.7. Statistical analysis

Statistical analysis was performed using SPSS 10.0 (SPSS Inc., USA). All data were shown as means ± standard deviation (SD). Comparisons between two groups were made using Student's independent-samples *t*-test, and for those with more than two groups, one-way analysis of variance (ANOVA) was performed followed by the Tukey tests used for the post hoc multiple comparisons between individual groups. The statistical significance level was set to be $p < 0.05$.

3. Results

3.1. HPLC–UV method development

Under our chromatographic condition, the injection of blank liposome lysis showed that the interference signals were negligible at the location of IS, DZNep, and PBA in the chromatogram (Fig. 1A). The elution time for ribavirin (IS), DZNep, and PBA was 1.76 ± 0.05 min, 2.28 ± 0.05 min, and 12.70 ± 0.25 min, respectively (Fig. 1B–D). The LLOQ for DZNep and PBA was determined as 0.5 μg/mL and 1 μg/mL (Fig. 1B and C), respectively. As shown in Table 1, the coefficient of determination (r^2) was greater than 0.99 in all *in vitro* samples. At the LLOQ, the RSD of both compounds was lower than 10% and the accuracy was within ±15% of the nominal values.

3.2. LogK_{ow} at pH 7.4 and stability of DZNep in weak acidic and neutral aqueous buffers at 60 °C

As shown in Table 2, within the concentration range of 5–50 μg/mL at pH 7.4, the experimental LogK_{ow} was −1.09 ± 0.01 in a concentration-independent manner ($p = 0.48$). In addition, after 50 μg/mL of DZNep was incubated in citrate buffer (pH 4.0) and 5% glucose solution (pH 7.0) at 60 °C for 2 h, the recovery of DZNep was found to be 96.5 ± 1.0% (pH 4.0) and 97.4 ± 1.2% (pH 7.0), respectively.

3.3. Optimization of the preparation method of L-DZNep by the determination of encapsulation efficiency

The encapsulation efficiency of DZNep within SUV liposomes using the REV method was 5.3 ± 1.0%, while that using the R-wo-PBA method was 6.9 ± 1.2% (Fig. 2). In contrast, the encapsulation efficiency of DZNep in L-DZNep reached 50.7 ± 2.9% using

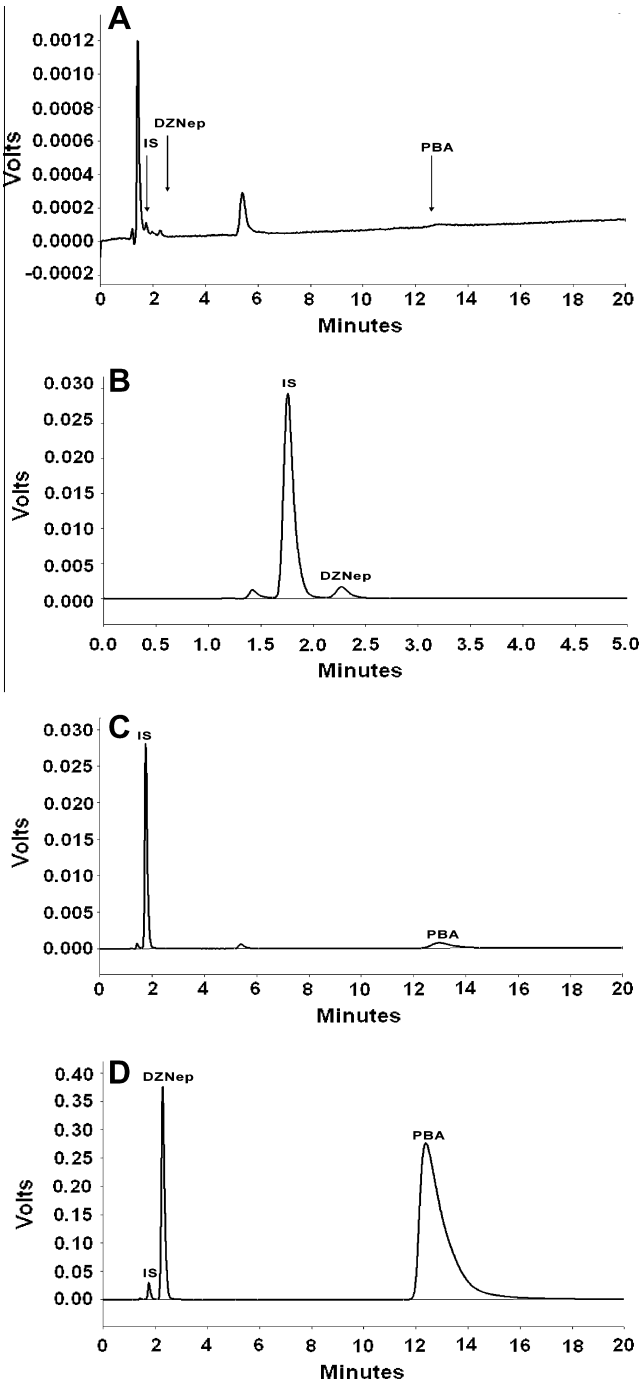

Fig. 1. HPLC–UV chromatogram of (A) a blank liposome lysis, (B) a blank liposome lysis spiked with DZNep at 0.5 μg/mL (LLOQ) and IS at 5 μg/mL, (C) a blank liposome lysis spiked with PBA at 1 μg/mL (LLOQ) and IS at 5 μg/mL, (D) a L-DZNep lysis spiked with IS at 5 μg/mL after drug loading and before removing unencapsulated DZNep and PBA by the dialysis.

the R-w-PBA method (PBA-to-DZNep ratio of 8:1), which was significantly higher than those using the other two methods ($p < 0.01$). As seen from Fig. 3, after five times dialysis against 5% glucose solution, the remaining PBA in the liposomes was 9.4 ± 4.5 μg/mL (0.33 ± 0.14% of initial input), while the remaining DZNep in the liposomes was 459 ± 35 μg/mL (50.7 ± 2.9% of initial input). As shown in Fig. 4, the encapsulation efficiency of DZNep in liposomes at PBA-to-DZNep ratio of 8:1 was 50.7 ± 2.9%, which was significantly greater than that (40.1 ± 2.2%) at the ratio of 6:1 ($p < 0.05$) but had insignificant difference with that (54.2 ± 1.8%)

Table 1
Validation characteristics of HPLC–UV method for assaying DZNep and PBA in the presence of blank liposome lysis.

	DZNep	PBA
Standard curve	$y = 0.0903x + 0.0689$	$y = 0.0574x - 0.5328$
Linear range	0.5–100 μg/mL	1.0–1000 μg/mL
Coefficient of determination (r^2)	0.9999	0.9994
LLOQ	0.5 μg/mL	1.0 μg/mL
Accuracy of LLOQ	86–108%	87–112%
Precision of LLOQ	8.7%	7.3%

Table 2
$\text{Log}K_{ow}$ of DZNep in PBS buffer (pH 7.4).

DZNep spiked (μg/mL)	50	10	5	p
$\text{Log}K_{ow}$	−1.10 ± 0.03	−1.10 ± 0.01	−1.08 ± 0.02	0.48
Mean of $\text{Log}K_{ow}$	−1.09 ± 0.01			

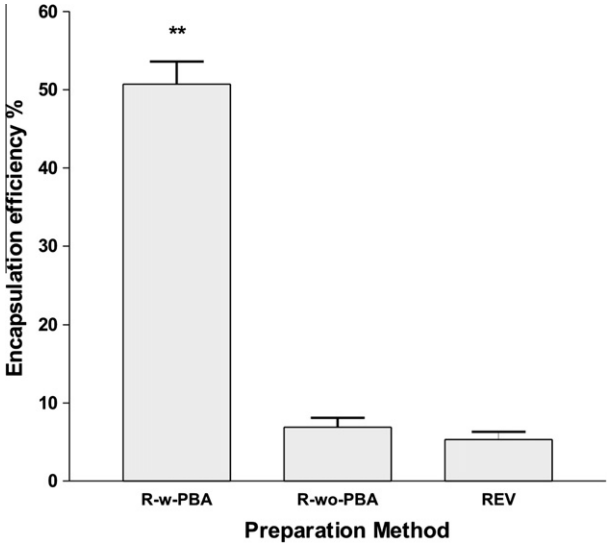

Fig. 2. Encapsulation efficiency of L-DZNep prepared using various methods (mean ± SD, $n = 3$). R-w-PBA, remote-loading in the presence of PBA; R-wo-PBA, remote-loading in the absence of PBA; REV, reverse-phase evaporation. $^{**}p < 0.01$ (R-w-PBA was compared with the other two methods).

at the ratio of 10:1 ($p = 0.64$). Thus, the optimum PBA-to-DZNep molar ratio of 8:1 was selected to produce the final L-DZNep. In addition, the encapsulation efficiency of DZNep in liposomes at PBA-to-DZNep ratio of 1:1 was 24.2 ± 4.7%.

3.4. Physicochemical characteristics of L-DZNep

The mean particle size of L-DZNep measured by Zetasizer 3000HS was 98.0 ± 2.7 nm ($n = 3$), and the polydispersity index was 0.20. As shown in Fig. 5, the L-DZNep appeared mostly spherical and uniform in size ranging around 100 nm as measured by a transmission electron microscopy (TEM). In addition, zeta potential of L-DZNep was −12.7 ± 0.6 mV in 5% glucose solution ($n = 3$) and −10.7 ± 1.2 mV in PBS solution ($n = 3$).

3.5. In vitro drug release

The *in vitro* release profiles of DZNep from pegylated liposomes and free drug solution (control) in both 5% glucose solution and rat plasma are shown in Fig. 6. The release of free DZNep in both

media was completed in 1 h. Approximately 30% and 45% of DZNep were rapidly released over a period of 6 h from L-DZNep in 5% glucose solution and rat plasma, respectively. After this initial burst, the DZNep was then released steadily, with the maximal release percentage at around 45% and 65% in 5% glucose and rat plasma, respectively. Total amount released from L-DZNep in rat plasma was significantly higher than that in 5% glucose ($p < 0.05$).

3.6. Pharmacokinetic studies of L-DZNep

The plasma concentration–time profiles of DZNep following intravenous bolus dosing of 1 mg/kg DZNep as free drug or pegylated liposomes in SD rats are shown in Fig. 7. The plasma DZNep concentration declined dramatically in the free drug group, which was lower than the effective level (622 ng/mL or 2.5 μM) after 10 min postdose [5] and finally fell below LLOQ (20 ng/mL) after 3 h postdose [17]. In contrast, after an identical dose of DZNep in liposomes group was administered to rats, the plasma DZNep concentration declined slowly and was maintained above the effective dose level up to 24 h and above the LLOQ for a period of at least 48 h postdose. The mean pharmacokinetic parameters of DZNep after intravenous administration of the two formulations in SD rats are summarized in Table 3. The area under the plasma concentration–time curve ($AUC_{0-\infty}$) was also markedly increased by approximately 138-fold in L-DZNep group (74.8 ± 9.0 h μg/mL) in comparison with the free DZNep group (0.54 ± 0.03 h·μg/mL). The plasma clearance of DZNep following the administration of L-DZNep (CL, 13.5 ± 1.6 mL/h/kg) to SD rats was substantially reduced by 99.3% as compared to that of the free DZNep (1850.1 ± 91.1 mL/h/kg). The steady-state volume of distribution (V_{ss}) of DZNep encapsulated in liposomes was found to be 0.13 ± 0.01 L/kg, which was about one-tenth of that of the free drug (1.34 ± 0.16 L/kg). Thus, the encapsulation of DZNep in pegylated liposome caused a 6-fold increase in the elimination half-life ($T_{1/2,z}$: 1.1 ± 0.1 h in the free DZNep versus 8.0 ± 1.4 h in the L-DZNep, $p < 0.01$) and a 13-fold increase in the mean residence time (MRT: 0.7 ± 0.1 h in the free DZNep versus 10.1 ± 1.5 h in the L-DZNep, $p < 0.01$) as compared to the free DZNep.

4. Discussion

The main purpose of this study was to improve the disposition characteristics of DZNep *in vivo* through the development of a

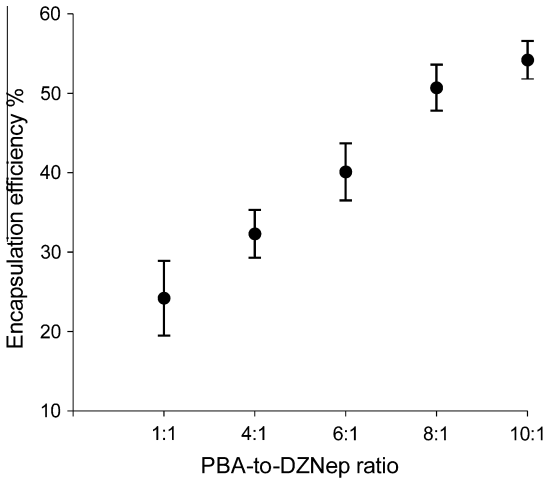

Fig. 4. Graph showing encapsulation efficiency of DZNep in liposomes against PBA-to-DZNep molar ratios of 1:1, 4:1, 6:1, 8:1, and 10:1. The ratio of DZNe-to-lipid was 1:10. The input DZNep concentration in the mixture was 1 mg/mL. Each value represents mean ± SD ($n = 3$).

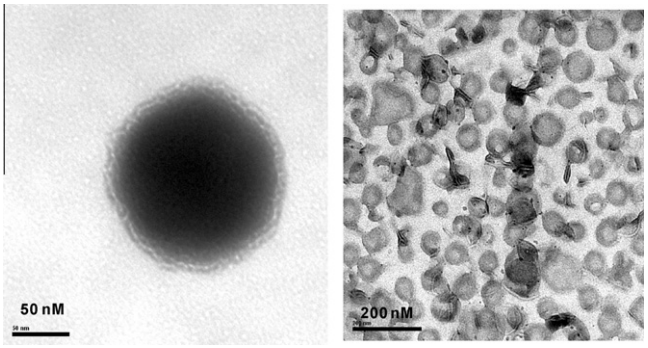

Fig. 5. Transmission electron microscopy (TEM) photograph of L-DZNep stained with 2% phosphotungstic acid solution. The liposomes obtained are relative homogenous in dimensions (right × 10 K, 100 kV) and at higher magnification (left × 50 K, 100 kV). All liposomes were produced by extrusion of multilamellar (MLV) liposomes through 0.1-μm polycarbonate membrane filters 10 times.

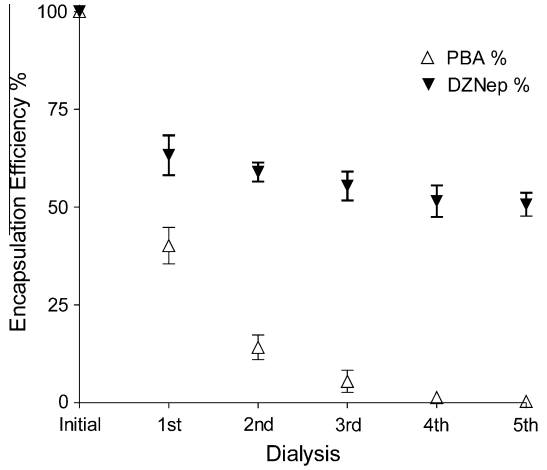

Fig. 3. Encapsulation efficiency of DZNep or PBA in liposomes-dialysis times profiles following the dialysis of coarse L-DZNep containing uncapsulated DZNep and PBA against 5% glucose solution (sink condition) 5 times, each for 1 h. Each value represents mean ± SD ($n = 3$).

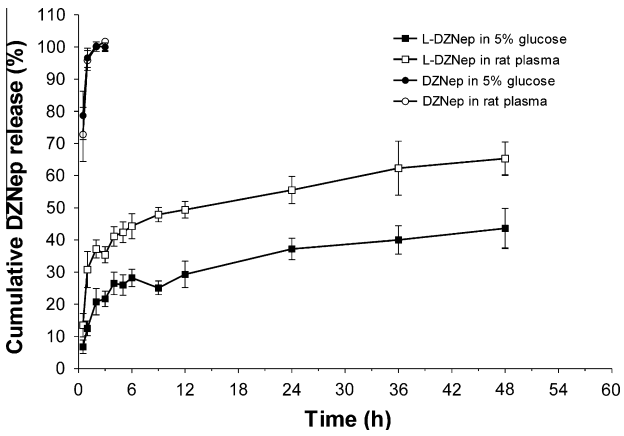

Fig. 6. *In vitro* cumulative DZNep release from L-DZNep (0.36 mg/mL) in 5% glucose (■) or plasma (□). *In vitro* cumulative DZNep release from free drug (0.36 mg/mL) inside dialysis bag in 5% glucose (●) or plasma (○) served as the control groups. Data are shown as mean ± SD ($n = 3$).

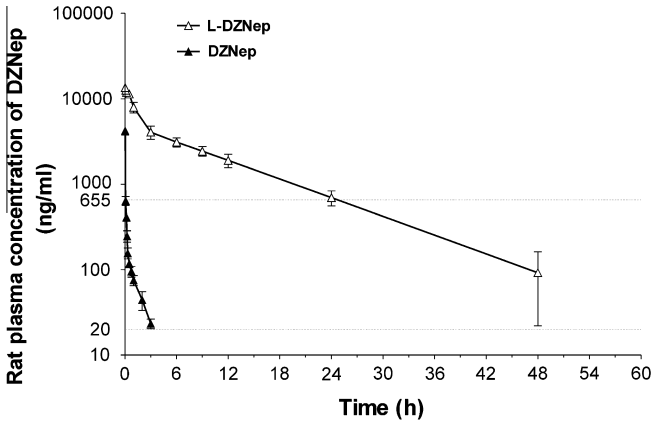

Fig. 7. Plasma DZNep concentration–time profiles following intravenous injection of DZNep solution (▲) and L-DZNep solution (△) in SD rats. The dose for two formulations was 1 mg/kg BW. Each value represents mean ± SD ($n = 6$). The lower dotted line indicates the LLOQ (20 ng/mL) of LC–MS/MS method for quantifying DZNep in the rat plasma. The upper dotted line indicates the effective level (655 ng/mL or 2.5 μM) that induced time-dependent cell death and reactivation of EZH2-related repressed genes in various solid tumors.

unilamellar pegylated liposomal formulation. A reliable and simple HPLC–UV method was developed for the quantification of DZNep and PBA in *in vitro* samples, except those containing rat plasma. As both DZNep and ribavirin are nucleoside analogs, sharing similar chemical structure and close hydrophilicity (ACD/Log*D*, pH 5.5: −2.21 for DZNep and −2.26 for ribavirin) in acidic condition, ribavirin was chosen as an IS in HPLC–UV method development. In addition, the HPLC–UV assay method for ribavirin [19] was employed and optimized for the quantification of DZNep *in vitro*. Although the HPLC–UV method was not as sensitive (e.g., LLOQ: DZNep, 0.5 μg/mL and PBA, 1 μg/mL; Table 1) as the LC–MS/MS method (e.g., LLOQ: DZNep, 20 ng/mL) [17], its application in the quantification of DZNep and PBA *in vitro* for liposome preparation was sufficiently adequate as the expected concentration range of DZNep was from 5 to 200 μg/mL, while that of PBA was from 1.5 to 750 μg/mL.

As the octanol–water partition coefficient (K_{ow}) best measures the behavior of a compound toward a membrane, it plays an important role in encapsulation efficiency of the compound in the liposomes [20]. It has been reported that the liposomes would favorably encapsulate drugs with a high LogK_{ow} value (>5) but not those with an intermediate LogK_{ow} value (1.7–4) [21,22]. Additionally, those with a low LogK_{ow} value (<−0.3) often possess the low entrapment and high leakage properties [21,22]. The concentration-independent LogK_{ow} of DZNep determined was −1.09 ± 0.01 (Table 2), indicating the high hydrophilicity of DZNep in a physiological condition. Thus, the conventional DZNep liposome would have the potentially low encapsulation efficiency and high leakage of DZNep if the conventional preparation methods such as the REV or the R-wo-PBA method were employed (Fig. 2). Moreover, the *in vitro* stability test showed that the content of DZNep remained constant in the weakly acidic and neutral aqueous solution at 60 °C. This ensures that DZNep would be highly stable for a L-DZNep preparation using the remote-loading method.

The REV method was initially employed as a formulation optimization for its known efficient way of encapsulating hydrophilic drugs [14]. To obtain a desired formulation size (around 100 nm), which is a key factor for the efficient extravasation of an antitumor vesicle through fenestrated endothelium of tumor blood vessel walls [8], the sequential extrusion at 60 °C through Nuclepore® Polycarbonate membranes with pore size of 0.2 μm and 0.1 μm was applied. However, the encapsulation efficiency of DZNep inside SUV liposomes was found to be as low as 5.3% (Fig. 2). Obviously, the REV method was ineffective for encapsulating DZNep. Possibly, the extrusion might induce heavy leakage of encapsulated DZNep by breaking lipid membrane and diminishing interior aqueous volume. In addition, a remote-loading procedure in the absence of PBA (R-wo-PBA) did not remarkably improve any encapsulation efficiency (6.9%) (Fig. 2). The hydrophilic property of DZNep, owing to the presence of three hydroxyl groups in cyclopentenyl sugar moiety, might considerably compromise the pH gradient driving force. In light of the purification mechanism of ribavirin containing vicinal hydroxyl groups (cis diol), which specifically bind to phenylboronic acid (PBA) at basic or neutral pH using Bond Elut PBA SPE columns [17], the remote-loading procedure was modified by incubating PBA and DZNep for 5 min before DZNep was added into blank liposomes. The remote-loading method in the presence of PBA (R-w-PBA) with the PBA-to-DZNep molar ratio of 8:1 was effectively optimized to obtain a significantly better encapsulation efficiency of DZNep (50.7%), as compared to both the REV and R-wo-PBA methods (Fig. 2), suggesting that PBA plays a key role in the accumulation of DZNep inside the liposomes. Furthermore, the encapsulation efficiency of DZNep in liposomes (24.2%) at the PBA-to-DZNep ratio of 1:1 was significantly lower than that at the ratio of 8:1 ($p < 0.05$). The reason may be the low chemical affinity between DZNep and PBA under the neutral condition. Thus, it is applicable to use higher PBA-to-DZNep ratio than the stoichiometry (1:1) to hasten the reaction process. A transient complex of PBA and DZNep could be produced in the neutral outer phase of the liposomes, which could readily enter the inner phase through the lipid bilayer [11]. Then, the complex would be spontaneously dissociated in the acidic environment as depicted by the chemical reaction in Fig. 8A. Due to the presence of a protonable amino group in the DZNep molecule (p*K*a = 6.22), the free DZNep would be protonized immediately and thus retained within the liposome interior.

As PBA is not a pharmaceutically acceptable additive, the riddance or reduction of the amount of PBA encapsulated in the liposomes is required. The five times dialysis method using 5% glucose solution appeared to reduce more selectively the amount of PBA (0.33% of initial input) than that of DZNep (50.7% of initial input) (Fig. 3). This might be due to the differences in p*K*a and LogK_{ow} between the two compounds. Without any protonable moiety, PBA always presents neutral form both inside and outside the liposomes. This enables PBA to readily and passively transport across the lipid membrane. Coupled with the intermittent ACD/LogK_{ow} (1.59), which is close to the intermediate partition coefficients ($1.7 < \log K_{ow} < 4$) [21,22], PBA would partition effortlessly between the lipid and aqueous phases. Therefore, PBA is a satisfactory shuttle in improving the encapsulation efficiency of DZNep while being easily washed off at the end of the dialysis.

Based on these findings in the liposomal preparation, the chemical reaction of the DZNep-PBA complex and the remote-loading model in the presence of PBA are summarized in Fig. 8A and B. Of notice, hydrophilic compounds with both a cis diol group and

Table 3
Pharmacokinetic parameters (mean ± SD) of DZNep after intravenous injection of DZNep solution or L-DZNep in six SD rats at the dose of 1 mg/kg BW.

Parameter	units	Free DZNep	L-DZNep**
λ_z	1/h	0.63 ± 0.14	0.09 ± 0.01
$T_{1/2,z}$	h	1.1 ± 0.2	8.0 ± 1.4
C_0	ng/mL	6692.0 ± 714.0	13811.2 ± 749.8
AUC_{total}	h ng/mL	541.6 ± 26.0	74762.1 ± 8952.8
Cl	mL/h/kg	1850.1 ± 193.1	13.5 ± 1.6
MRT	h	0.73 ± 0.19	10.1 ± 1.5
V_{ss}	mL/kg	1342.3 ± 162.2	134.8 ± 11.6

** $p < 0.01$ for all the PK parameters between two groups.

a p*K*a value of greater than 4, such as DZNep (p*K*a = 6.22), could be protonized and entrapped inside the acidic inner of liposomes using the remote-loading method. However, this proposed underlying mechanism may be different from Zalipsky's model [12], which deals with the other compounds containing a cis diol group, whose p*K*a values are less than 4, such as ribavirin (p*K*a = −1.59), riboflavin (p*K*a = 2.56), fluorouridine (p*K*a = −5.81), 8-chloroadenosine (p*K*a = 3.31), could not be protonized at the inner acidic condition (around pH 4.0) of liposomes prepared using R-w-PBA. Thus, one would expect that these compounds could not be firmly incorporated in the liposomes. The relatively high leakage of these compounds from the intraliposome would occur during storage.

The size and surface zeta potential of liposomes are key determinants for the level of the residence time of liposomes in blood, the transfer of liposomes from blood to tumor tissue interstitial spaces, the local retention of liposomes in the tumor tissue, and the efflux of liposomes from the tumor tissue to the blood [23]. Generally, the optimal size for elevating the level of tumor accumulation of the pegylated liposomes is around 100 nm [24]. The mean size of L-DZNep was 98.0 nm with the polydispersity index of 0.2, suggesting that L-DZNep possesses a satisfactory formulation size and a homogenous size distribution. The zeta potential of L-DZNep was found to be mildly negative at −10.7 mV in PBS solution (physiological condition), which has insignificant difference with that in 5% glucose ($p = 0.08$). This result suggests that ions in physiological condition may have little effect on the zeta potential of L-DZNep. Although charged liposomes demonstrate a better physical stability than neutral liposomes while being stored

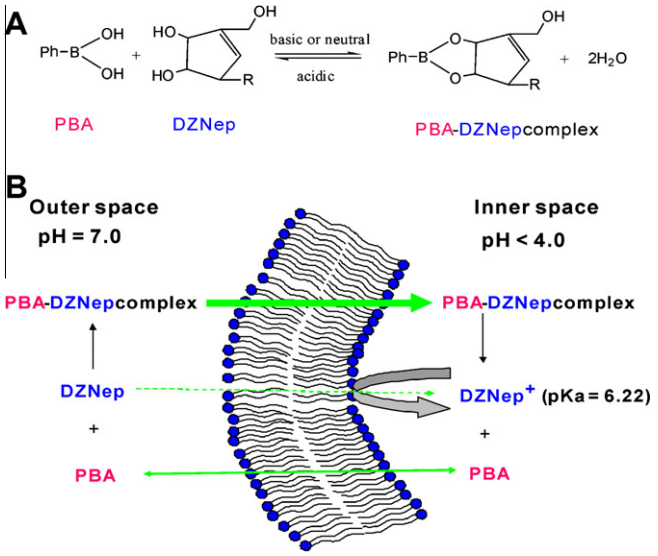

Fig. 8. (A) A chemical reaction involved in the product of the reversible DZNep–PBA complex through the dehydration in the external solution of the liposomes. (B) Schematic representation of diffusion and entrapment mechanism of DZNep in the presence of PBA. In the beginning, a transmembrane pH gradient (neutral outer phase and acidic inner phase) is formed through the dialysis to remove the outer ammonium sulfate. A transiently stable lipophilic DZNep–PBA complex in the neutral external solution is generated, which is capable of diffusing across the bilayer. As for the free DZNep in the outer space, the diffusion rate is limited by the hydroxyl groups in cyclopentenyl sugar moiety. Dissociation of the complex occurs in the acidic inner compartment of liposomes. Due to the presence of a protonable amino group in the DZNep molecule (p*K*a = 6.2), the dissociated DZNep is ionized at once in the acidic condition. The protonized form of DZNep is impermeable to the liposome lipid bilayer and thus remains entrapped in the liposomes. As PBA cannot be protonized readily and possesses an intermediate LogK_{ow} (1.6), it could easily traverse the lipid bilayer and return to the exterior space of liposomes to shuttle another drug molecule. For the same reason, PBA can be easily removed at the end of the experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[25], the negatively charged liposomes tend to have a short elimination half-life in blood than their neutral counterpart [26].

The release of free DZNep in both 5% glucose solution and rat plasma was completed around 1 h, indicating that drug penetration through dialysis membrane was not a limiting step (Fig. 6). The rapid release of DZNep over a period of 6 h from L-DZNep in both the media (Fig. 6) was possibly due to the leaked DZNep during storage or the liposomes with small diameters (e.g., around 50 nm). A larger curvature of these liposomes might result in the release of the drug more readily [23]. DZNep released from L-DZNep was significantly increased in the presence of rat plasma ($p < 0.05$) (Fig. 6), indicating that the plasma protein could speed up the leakage of DZNep from the liposomes. Two possible mechanisms could account for the enhanced leakage: (i) the interaction of liposomes with serum proteins leads to the transfer of liposomal lipids to serum lipoproteins [27] and (ii) the insertion of complement components results in the formation of a 10-nm-diameter pore-like membrane attack complex (MAC) [28]. Besides, serum proteins have been implicated in opsonization, i.e., the process of binding of serum proteins, such as complement components, which promote the recognition and uptake of foreign particles by the mononuclear phagocyte system (MPS) [29,30]. Thus, the release of contents in liposomes is facilitated *in vivo*, but not in *in vitro* conditions, suggesting that *in vitro* release study can only partially predict *in vivo* drug release.

Since the two plasma concentration–time profiles of free DZNep and L-DZNep were best described by the three-compartment and two-compartment disposition models (data not shown), respectively, the non-compartmental PK analysis was performed for comparing the PK parameters of these two formulations. In comparison with the free drug, encapsulation of the DZNep in pegylated liposome markedly increased AUC as a result of the 99.3% reduction of the plasma clearance and 90% reduction of the steady-state volume of distribution and thus increased the plasma elimination half-life by 6-fold and the mean residence time by 13-fold (Table 3). The steady-state volume of distribution of the drug encapsulated in liposomes (0.13 L/kg) was significantly less than the total tissue water (0.79 ± 0.012 L/kg) in rats [31], suggesting that L-DZNep is largely confined between the systemic circulation and the extracellular space in the body. Therefore, this liposome formulation could effectively prolong the retention of DZNep in the systemic circulation. The more liposomes circulate in blood for a longer period, the greater their accumulation in tumor may occur [23]. Accordingly, this formulation (L-DZNep) is highly likely to increase the DZNep's tumor localization as well as to improve the drug therapeutic index in further preclinical studies with animal models bearing human tumor xenograft.

5. Conclusion

In summary, a reliable and simple HPLC–UV method for the quantification of DZNep and PBA in *in vitro* samples (except those containing rat plasma) was developed. DZNep is a hydrophilic compound and highly stable in the weakly acidic and neutral aqueous solution at 60 °C. The remote-loading method in the presence of PBA was effectively optimized to obtain an appropriate encapsulation efficiency of DZNep, a satisfactory vesicle mean size, a homogenous size distribution, a mildly negative zeta potential value, and a sustained-release property for DZNep *in vitro*, all of which suggest that the L-DZNep is a suitable candidate for the *in vivo* delivery of DZNep to rats via intravenous injection. The entrapment of DZNep within a pegylated liposomal carrier noticeably ameliorated the pharmacokinetic parameters of DZNep, including the marked increase in the distribution half-life, the elimination half-life, the initial plasma concentration, the mean

residence time, and the area under the concentration–time curve, as a result of substantial decrease in the plasma clearance and the steady-state volume of distribution. Therefore, the liposome formulation could effectively prolong the retention of DZNep in the systemic circulation and hopefully improve the DZNep's tumor localization and the drug therapeutic index in future preclinical and clinical studies. This study also provides a valuable example for the liposomal development of the hydrophilic nucleoside analogs containing a cis diol group and a p*K*a value of greater than 4.

Acknowledgements

This research was supported by the National University of Singapore Academic Research Fund R148-000-092-112. The authors are indebted to Mr. Wenji Li for technical support in animal work.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2011.10.014.

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