

A novel polyrotaxane-based intracellular delivery system for camptothecin: *In vitro* feasibility evaluation

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Abstract: Camptothecin (CPT) is a naturally occurring alkaloid that shows promise in antitumor activity *in vitro* against various tumor cell lines. Its potential clinical uses, however, are hindered by a lack of reaction selectivity and poor water solubility. Presented herein is a novel polyrotaxane (PR)-based delivery system that could potentially lead to a highly effective yet less toxic CPT therapy. The approach involves the synthesis of the PR-CPT conjugates via hydrolyzable linkages. To enhance the therapeutic efficacy of CPT, a cell-penetrating peptide, LMWP, is linked to the conjugate to allow specific, intratumoral delivery of CPT. To avoid nonselective uptake of the conjugates by normal tissues following administration, the cell-penetrating function of LMWP on the conjugates is masked by heparin binding. This system was designed such that after accumulation at the tumor via the enhanced permeability and retention (EPR) effect, protamine can be subsequently

administered to unmask heparin inhibition on LMWP, permitting intracellular uptake of the LMWP-PR-CPT conjugates. Once inside the tumor, CPT molecules are detached from the PR chain by hydrolysis, yielding a sustained concentration of CPT within tumor cells. In this paper, we demonstrated the *in vitro* feasibility of this delivery system. The LMWP-PR-CPT conjugates yielded a sevenfold increase in the overall CPT solubility, as well as a sustained release of CPT over a period of more than 7 days. Intracellular uptake of these conjugates by A2780 human ovarian cancer cells and regulation of such uptake by heparin and protamine were tested by MTT assay and confocal/flow cytometric methods, respectively. © 2007 Wiley Periodicals, Inc. *J Biomed Mater Res* 84A: 238–246, 2008

Key words: camptothecin; polyrotaxane; low molecular weight protamine; heparin inhibition

INTRODUCTION

Many anticancer drugs that are effective *in vitro* may not be effective *in vivo* because of undesirable physicochemical behavior (e.g., poor solubility) and/or toxic side effects. Since all of the intrinsic properties of a drug are fixed after synthesis, the only means to overcome such problems and thereby improve the clinical applicability of the drug is via the design of an appropriate delivery system. Camptothecin (CPT) represents a typical example of such

antitumor agents. It is a naturally occurring alkaloid isolated from the Chinese tree *Camptotheca acuminata*, with promising antitumor activity against various leukemia cell lines.¹ The intact lactone structure of CPT forms a noncovalent complex with topoisomerase I and DNA, inhibiting the resealing process of the broken DNA backbone and thus yielding antitumor effects. However, because of a lack of reaction selectivity, the use of CPT can cause severe side effects such as gastrointestinal toxicities. In addition, CPT is poorly soluble in aqueous media,² thereby limiting its effectiveness in clinical applications. Because of these restrictions, further investigation of CPT as an anticancer agent was discontinued in the 1970s. More recently, however, several modified CPT derivatives possessing increased solubility and reduced toxicity have been developed. Among these derivatives, irinotecan (CPT-11) and topotecan (TPT) have indeed already been approved for the treat-

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ment of colorectal and ovarian cancers. Unfortunately, in aqueous solutions the lactone ring of these CPT derivatives undergoes a rapid, reversible, and pH-dependent hydrolysis, yielding an open-ring carboxylate structure that has far less potent anticancer effects, and also presents a risk of causing hemorrhagic cystitis.³ The quest for a delivery system to improve the clinical efficacy of CPT thus continues.

During tumor angiogenesis, the nascent capillaries supplying nutrients to the tumors possess large gaps in between vascular endothelial cells when compared to healthy tissues. These gaps render the blood vessels of the tumor permeable to macromolecules (>30 kDa), whereas the capillaries in normal tissues typically do not allow these large molecules to traverse. Furthermore, the macromolecules tend to collect and accumulate in the interstitial space of the tumor due to the lack of a developed lymphatic drainage system. This phenomenon is known as the enhanced permeability and retention (EPR) effect,⁴ and has been widely exploited as a passive means for tumor targeting with macromolecular drug carrier systems.^{4–6}

The use of supramolecular assemblies of biocompatible and biodegradable polyrotaxane (PR) as a drug delivery vehicle has recently drawn great interest.⁷ The name “rotaxane” comes from the Latin words for wheel and axle, and thus the term “polyrotaxane” refers to a molecular assembly in which several cyclic molecules (e.g., α -cyclodextrin; α -CD) are threaded onto a linear polymer (e.g., PEG) chain. Bulky blocking groups (e.g., tyrosine) are then introduced at the ends, as caps to prevent the PR from dethreading. Small drug molecules can be linked to the abundant —OH groups on the CD molecules by hydrolyzable (e.g., ester) bonds, thus allowing for a sustained release of the attached drugs over a prolonged period. In addition, the bulky caps can also be linked with a cleavable bond to permit eventual dethreading of PR into its constituents of CD and PEG; both are biocompatible and can be readily cleared from the body.^{8,9}

Because of the impermeable nature of the cell membrane, many drugs, particularly large and hydrophilic agents, cannot internalize tumor cells to exert their therapeutic effects. In this regard, the recent discovery of the class of cell-penetrating or protein transduction domain (PTD) peptides has produced tremendous interests in drug delivery research, because it may now be possible to resolve the issue of poor cellular uptake by many drugs. Several PTD peptides have been identified, including TAT from human immunodeficiency virus (HIV-1),¹⁰ VP22 from herpes simplex virus,¹¹ ANTP homeotic transcription factor from *Drosophila Antennapedia*,¹² certain synthetic arginine-rich peptides,¹³ and the low molecular weight protamine (LMWP) peptide

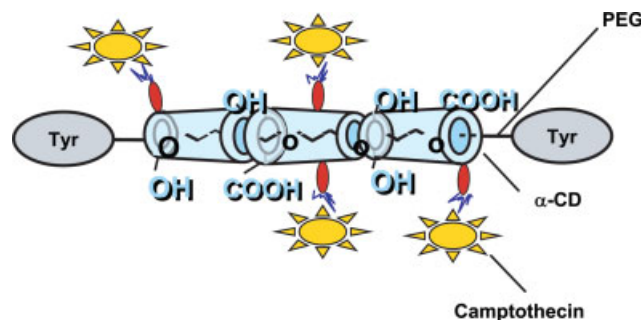


Figure 1. Schematic illustration of the CPT–PR conjugates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

developed in our laboratory by enzymatic digestion from native protamine.^{14–17} Both *in vitro* and *in vivo* studies have shown that, by covalently linking PTDs to almost any species, including large proteins ($M_w > 150$ kDa) and nanocarriers, these PTDs are able to ferry the attached species across cell membranes of all organ types including the brain.^{10,18} This PTD-mediated cell entry has been shown to be receptor independent, and appears to require a surface adsorption step, likely via binding to the anionic heparin sulfate molecules on the cell surface, since PTD-mediated cell uptake was completely inhibited by the presence of heparin.^{10,13} This PTD-mediated cell entry is kinetically far more efficient compared with existing uptake methods, such as receptor-mediated endocytosis.¹⁷ Yet, the absence of target selectivity in cell entry renders this method an unacceptable practice, due to concerns of drug-associated toxic side effects.

Proposed herein is a novel PR-based carrier system for effective delivery of CPT (Fig. 1). To further enhance the therapeutic efficacy of CPT and reduce its toxic effect to the surrounding tissues, a cell-penetrating peptide is linked to the PR–CPT conjugate to allow for tumor-specific, intracellular delivery of CPT. In order to avoid nonselective uptake of the conjugates by normal tissues following administration, the transmembrane function of LMWP on the conjugates is masked by binding with heparin. Finally, after accumulation of the conjugates at the tumor site by EPR-mediated passive targeting, protamine sulfate, a clinical heparin antagonist that binds to heparin with greater affinity than do LMWP, can be subsequently administered to unmask LMWP, thereby permitting a highly effective targeted intracellular uptake of the PR–CPT–LMWP conjugates. Once inside tumor cells, CPT can detach from the PR polymer chain by hydrolysis, yielding a sustained level of the CPT within the tumor cells.

In the present study, we conducted *in vitro* cell culture studies to demonstrate the feasibility of this

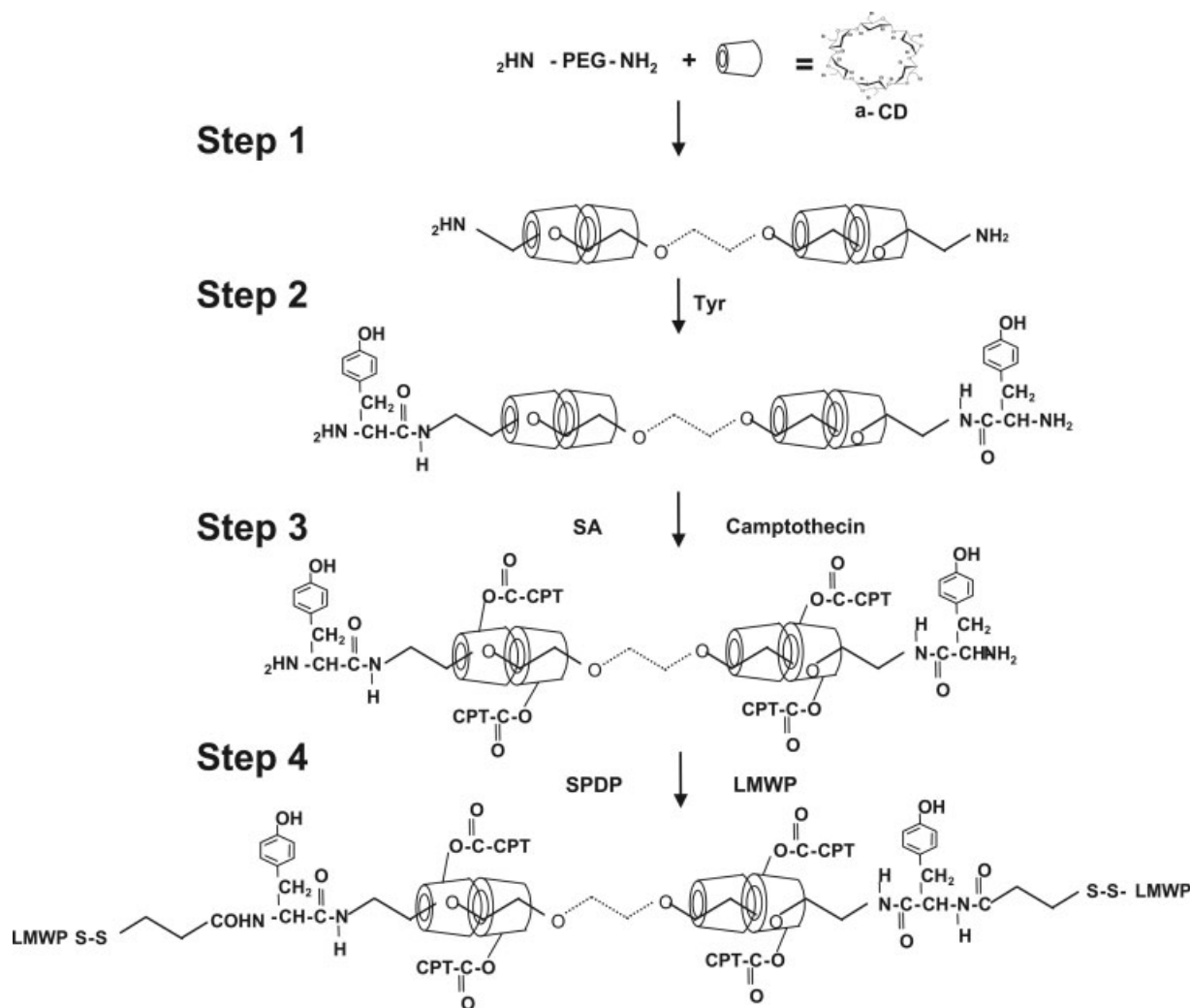


Figure 2. Schematic illustration of the synthesis of CPT-PR-LMWP conjugates.

CPT delivery system. The PR-CPT-LMWP conjugates were successfully synthesized and characterized. Furthermore, the controlled regulation of intracellular uptake of these conjugates using heparin and protamine was demonstrated.

EXPERIMENTAL PROCEDURES

Materials

Boc-L-tyrosine hydroxysuccinimide ester (BOC-Tyr-OSu) was from purchased Fluka (Allentown, PA). Unless otherwise stated, all chemicals including (S)-(+)-CPT, (polyoxyethylene glycol)bisamine (PEG-BA; average M_n : 3350 Da), and α -CD were purchased from Sigma Chemical (St. Louis, MO). All solvents were purchased from Aldrich Chemical (Milwaukee, WI). Water was distilled and deionized (ddH₂O).

Synthesis of LMWP-PR-CPT (LMWP-PR-CPT) conjugates

A schematic illustration of the synthesis of the LMWP-PR-CPT conjugates is shown in Figure 2. Pseudo-PR was first prepared according to the procedures of Harada and Kamachi.¹⁹ In brief, 5 mL of solution containing 1 g PEG-BA (3.0×10^{-4} mol) was added to a saturated solution of α -CD (12 g, 1.2×10^{-2} mol), and the mixture was ultrasonically agitated for 1 h followed by stirring for 24 h at room temperature. Precipitated pseudo-PR was collected by centrifugation, dried under vacuum, and then stored at room temperature for subsequent use (see Step 1 in Fig. 2).

To synthesize the PR polymer, Boc-Tyr-OSu (5 g, 1.3×10^{-2} mol in DMSO) was added to a DMSO solution containing 12 g (4×10^{-4} mol) of the pseudo-PR, and the reaction mixture was stirred at room temperature for 96 h. The Tyr-capped PR polymer was precipitated by the addition of excess acetone, collected by centrifugation, washed with ethanol and water, and then dried at room temperature (Step 2).

To prepare the PR-CPT conjugates, the Boc-Tyr-terminated PR (1 g, 3.0×10^{-5} mol) was first activated with succinic anhydride (SA; 5 g, 5.0×10^{-2} mol in pyridine), and the mixture was stirred at room temperature for 72 h. CPT (350 mg, 1.0×10^{-3} mol) was then added to a toluene solution (50 mL) containing 400 mg (1.0×10^{-5} mol) of the activated PR carboxyethyl ester, and the solution was azeotroped for 2 h. The solvent was removed by vacuum, and pyridine (50 mL) was added to dissolve the precipitate. The solution was then cooled to 0°C for 15 min, followed by the addition of 2-chloro-1-methylpyridinium iodide (530 mg, 2.0×10^{-3} mol) and 4-dimethylaminopyridine (DMAP) (500 mg, 4.1×10^{-3} mol). The mixture was stirred at room temperature for 96 h, and the product was precipitated by the addition of ether. Removal of the Boc groups was carried out by following a well-established procedure described by Han et al.²⁰ The PR-CPT product was collected by precipitation with ether, purified by washing with 2-propanol, and then subjected to ¹H NMR (model no. DRX-500; Bruker Biospin, Billerica, CA) using DMSO-*d*₆ as the solvent for structural analysis (Step 3).

To synthesize the LMWP-PR-CPT conjugate, LMWP was first prepared by digestion of native protamine with thermolysin, according to previously established procedures.¹⁴⁻¹⁷ The completely purified LMWP consists of 14 amino acids with a sequence of SRRRRRRGGRRRR.

Conjugation of LMWP to PR-CPT was carried out using a modified procedure by Yang and coworkers¹⁷ (Step 4).

Fluorescence labeling

Twenty microliters of the Lissamine rhodamine B ethylenediamine (Rho) dye, prepared in DMSO at a concentration of 10 mg/mL, was mixed with the drug conjugates (PR-CPT or LMWP-PR-CPT) in labeling buffer (0.24M methylimidazole, pH 9.0, 0.32M [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride], EDC) at a final CPT concentration of 2 μM. The mixture was reacted at room temperature for 4 h, and fluorescence-labeled drug conjugates were separated by size exclusion chromatography.

Determination of CPT content in the conjugates

To determine the CPT content in the PR-drug conjugates, ~2 mg of the conjugates were placed in a volumetric flask containing 20 mL of a 1:1 mixture of 50 mM borate buffer (pH 9.5)/methanol solution. The flask was shaken vigorously at 37°C for 24 h, and the reaction was quenched by the addition of concentrated H₃PO₄. The solution was then analyzed using HPLC, with a C-18 Microsorb-MV column (Rainin, Walnut Creek, CA) and an isocratic mobile phase consisting of 25% (v/v) acetonitrile and 2% (w/v) triethylamine acetate. The lactone and carboxylate peaks in CPT were determined by the areas under the absorbance curves at 254 nm at the 5- and 16-min marks, respectively, from the HPLC chromatograms and were then compared with the CPT standards.

Solubility determination

The solubility of free CPT and PR-bound CPT were determined by adding the drug samples to vials containing 50 mM phosphate buffer (pH 7.4). The vials were incubated with agitation, in a thermal bath at 37°C for 24 h, and insoluble materials were filtered with 0.45-μm-disc filters. The CPT content in the solutions of these samples was then diluted with methanol and analyzed using HPLC.

In vitro drug release studies

The release of CPT from the PR-drug conjugates by hydrolysis was evaluated at 37°C in phosphate buffer (pH 7.4) containing 0.02% Tween 80. Approximately 1 mg of the PR-CPT conjugates was suspended in 1 mL of PBS, and then placed in a dialysis bag (molecular weight cutoff: 3000 Da). The mixture was dialyzed against 30 mL PBS at an agitation rate of 100 rpm, and 1 mL of the buffer solution was collected at designated time intervals and analyzed by HPLC. An equivalent volume of fresh buffer was added to the release buffer after each sample withdrawal.

In vitro cytotoxicity

CPT-induced cytotoxicity was assessed by a calorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In brief, 1×10^5 A2780 human ovarian cancer cells (ECACC, Salisbury, UK) were seeded into 96-well plates. After 24 h incubation at 37°C, the medium was aspirated and 10 different concentrations of sterile test substances (free CPT, PR-CPT, and LMWP-PR-CPT) in fresh media were added. Control cells were treated with an equivalent volume of Dulbecco's phosphate buffered saline (DPBS). Cells were subsequently cultured for 48 h, the medium was aspirated, and 100 μL of fresh medium and 25 μL of 5 mg/mL MTT solution were added to each well. The plates were incubated for 4 h at 37°C and formazan crystals were dissolved by the addition of DMSO. Finally, absorbance was measured at 570 nm.

Cell uptake assays

A2780 cells were plated on 4-well chamber slides at a density of 1×10^4 cells/slide and incubated at 37°C in a humidified atmosphere of 5% CO₂. After complete adhesion, the cell medium was removed. Five test samples, containing Rho-labeled CPT, including: (1) free CPT; (2) PR-CPT; (3) LMWP-PR-CPT; (4) LMWP-PR-CPT + heparin; and (5) LMWP-PR-CPT + heparin + protamine were added to the cells at a final CPT concentration of 2 μM. Following incubation for 2 h at 37°C, cells were washed with PBS, fixed with 1% paraformaldehyde, and visualized using confocal laser scanning microscopy (Carl Zeiss, Gottingen, Germany).

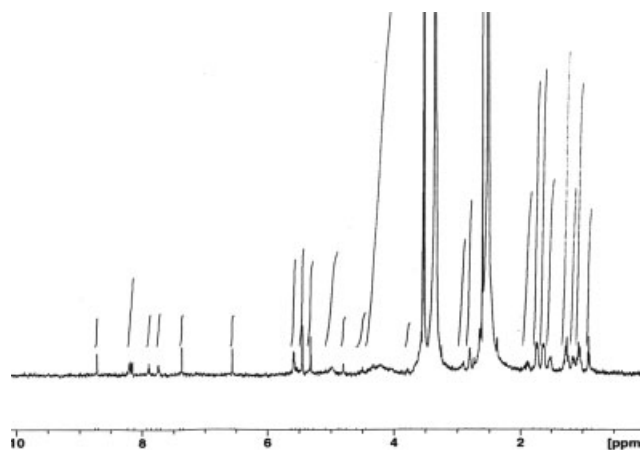


Figure 3. NMR spectrum of the synthesized CPT-PR conjugates.

Flow cytometric analyses

A2780 cells were plated on 6-well plates at a density of 1×10^6 cells per well and incubated at 37°C in humidified atmosphere of 5% CO₂ for 24 h. The cells were then washed with PBS and incubated with the five previously described test samples at a final CPT concentration of 2 μM for 2 h. Cells were washed with PBS to remove surface bound drug conjugates and treated with trypsin-EDTA to collect the cells. The cells were then fixed with 1% paraformaldehyde and washed with PBS. Analysis was conducted on a FACScalibur flow cytometer (Beckton Dickinson, San Jose, CA) equipped with a 488-nm air-cooled argon laser.

RESULTS

Synthesis and characterization of the PR-CPT conjugates

As described previously, the PR-CPT conjugates were synthesized by threading the α-CD residues onto a PEG chain, blocking the two ends of the PEG chain with bulky L-Tyr groups, activating the resulting PR polymer with SA, and then linking CPT on the SA-activated groups via hydrolysable ester linkages. Figure 3 shows the NMR spectrum of the synthesized PR-CPT conjugates, with peaks representing ¹H NMR (DMSO): δ (ppm) = 0.9 (t, C(18)H₃ of CPT), 1.9 (m, C(19)H₂ of CPT), 2.3 (s, CH₂ of CEE), 2.9 (m, CH₂ of Tyr), 3.5 (s, CH₂ of PEG), 5.5, 5.6 (dd, OH(2), OH(3) of CD), 8.3 (q, C(12) H₁ of CPT). As demonstrated by the related peaks for CPT, CD, Tyr, and PEG, it was clear that the desired PR-CPT conjugates were successfully synthesized. The overall yield of the PR-CPT conjugate was 56%, whereas the average weight content of CPT in the conjugate was ~8.0% (w/w). The molecular weight of the PR-CPT conjugate, as determined by the ¹H NMR data,

was approximately 45 kDa. Based on these results, each PR-CPT conjugate was calculated to possess one PEG chain, 30 α-CD residues, and 10 CPT drug molecules.

When compared to weight calculations of CPT, the solubilities of free CPT and the PR-CPT conjugate were estimated to be 28.3 and 195.0 μg/mL, respectively. This sevenfold increase in CPT solubility by the PR-CPT conjugate was apparently influenced by both the hydrophilic nature of the polymer constituents (PEG and CDs) as well as the presence of abundant -COOH groups resulting from activation of the -OH groups on the CD residues by SA.

The release of CPT by hydrolysis from the PR-CPT conjugates was also examined *in vitro* under physiological conditions (Fig. 4). Within first 5 h of incubation, a burst release of CPT was observed, with less than 20% of total CPT bonds hydrolyzed. After the initial effect, a nearly linear and sustained release profile of the drug was seen over an extended period. By day 7, more than 75% of CPT on the polymer-drug conjugates had been released by hydrolysis.

In vitro cytotoxicity studies

Cytotoxicity of the PR-drug conjugates were examined *in vitro* against the A2780 human ovarian cancer cell line using the MTT method. The ID₅₀ values for free CPT, PR-CPT, and LMWP-PR-CPT, as determined from the MTT cytotoxicity assay (Fig. 5), were 0.006, 1.41, and 0.13 μM, respectively. While the LMWP-PR-CPT conjugate was about 20-fold less potent in killing cancer cells than free CPT, it nevertheless yielded a 10-fold increase in the cyto-

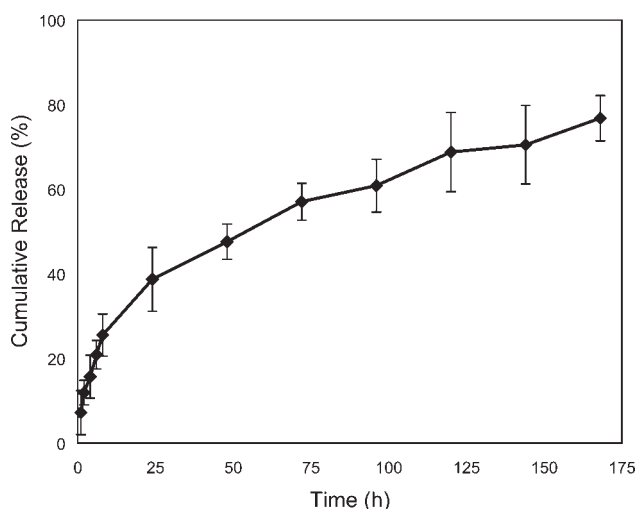


Figure 4. CPT release from CPT-PR conjugate in PBS (pH 7.4).

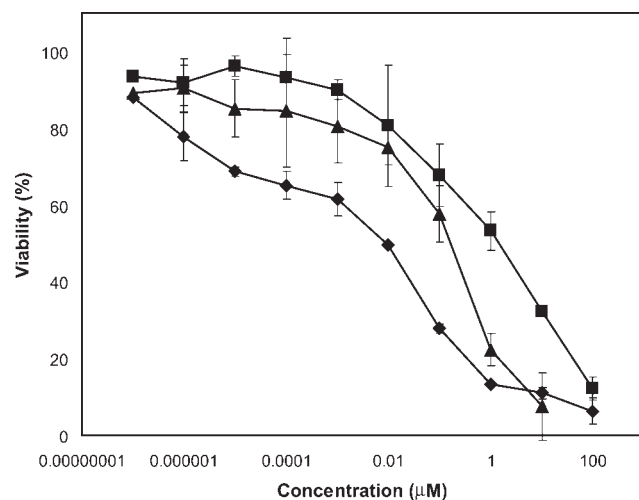


Figure 5. Cytotoxicity of CPT and CPT-polymer conjugates. Free CPT (◆), CPT-PR (■), and CPT-PR-LMWP (▲) conjugates against A2780 human ovarian cancer cells in log-phase culture. Values represented as mean \pm SD. Each experiment was performed in triplicate.

toxic effect over that of the conjugate without attachment of the cell-penetrating LMWP peptide.

LMWP-mediated intracellular delivery of CPT and regulation by heparin and protamine

CPT exhibits its own blue fluorescence with excitation and emission wavelengths of 370 and 435 nm, respectively. To monitor LMWP-mediated intracellular delivery of the conjugates, the PR chain was labeled with Rho (excitation at 570 nm and emission at 590 nm) on the carboxylic acid groups. When free CPT was added, the small and hydrophobic CPT molecules were able to diffuse into the cells with ease following 2 h of incubation, as reflected by the high intensity of blue fluorescence within the exposed cells (Fig. 6.1). On the other hand, the PR-CPT conjugates were not taken up by the carcinoma cells (Fig. 6.2), primarily due to the macromolecular structure of the PR polymer. Conversely, the LMWP-PR-CPT conjugates were able to internalize into the cells, as the blue CPT-derived fluorescence and red fluorescence from Rho-labeled PR were superimposed inside cells including colocalization (Fig. 6.3). These results indicated that the cell-permeable LMWP was able to carry the attached PR-CPT conjugates across cell membranes. When heparin was added, this LMWP-induced cell transduction function was markedly abolished (Fig. 6.4), likely due to the electrostatic interactions between the positively-charged LMWP and negatively-charged heparin molecules. Yet, when protamine was added to the mixture, the heparin-induced inhibition was nearly completely reversed (Fig. 6.5), as demonstrated by recovery of CPT (blue) and the PR chains

(red) of fluorescence colocalization within the cells. Consistent with these findings, flow cytometric analysis indicated that LMWP was able to ferry the cell-impermeable PR-CPT conjugates into the carcinoma cells (Fig. 7). Similarly, this LMWP-mediated intracellular uptake was inhibited by the addition of heparin but was recovered by the addition of protamine to the heparin-masked LMWP-PR-CPT conjugates.

DISCUSSION

Most potential antitumor agents including CPT face a major hurdle in their clinical applications, due to a lack of target selectivity for their therapeutic actions. While drug action against tumor cells would result in therapeutic efficacy, actions on normal tissues would consequently lead to toxic side effects. To counter this problem, the use of polymer-based carriers for delivery of such small hydrophobic agents have been extensively explored and actively pursued; simply because these nanocarrier systems would be able to accumulate within tumors based on the EPR-mediated passive targeting. Among the various polymer carrier systems, the unique PR supramolecular assembly offers an appealing platform for drug delivery due to several key advantages. First, the two primary building blocks of PR, α -CD, and PEG are both widely used throughout the pharmaceutical industry and are considered safe and relatively nontoxic. In addition, the large number of hydroxyl groups on the α -CD residues of PR can be easily modified and function as multivalent drug carriers and also used to improve the physicochemical properties of the attached drug molecules, such as solubility and stability. Furthermore, both the size and molecular weight of the PR carrier can be readily regulated, either by altering the chain length of PEG or by changing the molar ratio between α -CD and PEG during the threading process, to produce the most desirable platform in avoiding renal filtration and reticuloendothelial system clearance. Similarly, the ability to modify the size of the PR carrier also facilitates the EPR-induced passive tumor targeting. According to our results, the synthesized PR possessed a molecular weight of 40–45 kDa, as determined NMR and confirmed by gel filtration chromatography by utilizing poly (2-vinylpyridine) and certain standard proteins as molecular weight markers. Using ^1H NMR analysis, the number of α -CD molecules inserted onto the PEG chain and the SA-activated carboxyethyl ester groups were estimated to be 30 and 100, respectively. The average molar ratio of ethylene oxide/CD was 2.5, which was in reasonable agreement with the theoretical value of 2.0–2.2 suggested by other investigators.^{8,19} Nevertheless, CPT

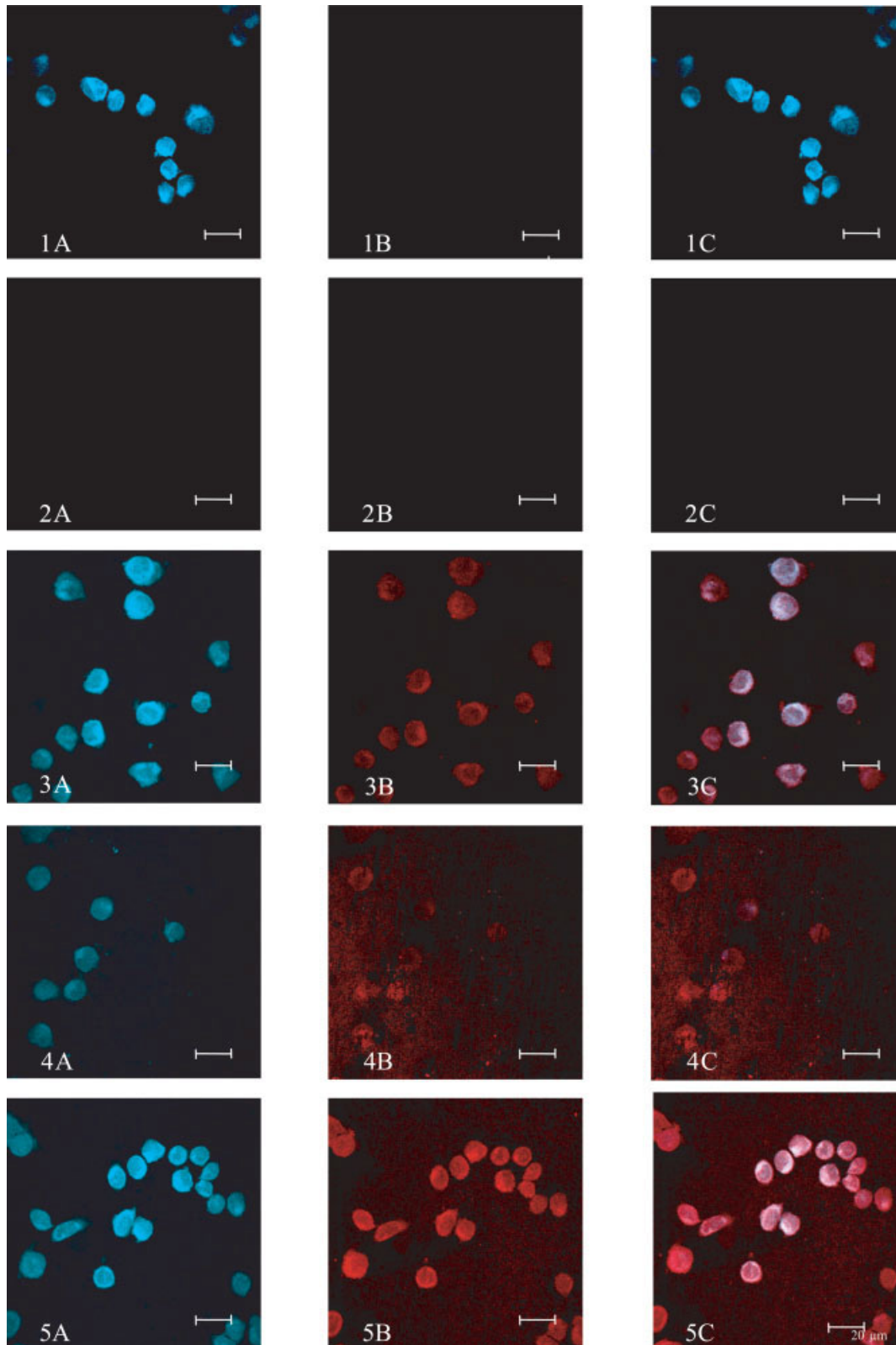


Figure 6. Cellular localization of Rho-labeled LMWP–drug conjugates in A2780 human ovarian carcinoma cells. Free CPT, Rho-labeled CPT–PR, Rho-labeled CPT–PR–LMWP, Rho-labeled CPT–PR–LMWP and heparin mixture, and Rho-labeled CPT–PR–LMWP, heparin, and protamine mixture were overlaid onto cultured A2780 cells in the presence of 10% FBS. Cellular localization was monitored by confocal microscopy. (1) Free CPT; (2) Rho-labeled CPT–PR; (3) Rho-labeled CPT–PR–LMWP; (4) Rho-labeled CPT–PR–LMWP and heparin mixture; (5) Rho-labeled CPT–PR–LMWP, heparin, and protamine mixture. (A) 430 nm (blue) detection; (B) 560 nm (red) detection; (C) overlaid (A + B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

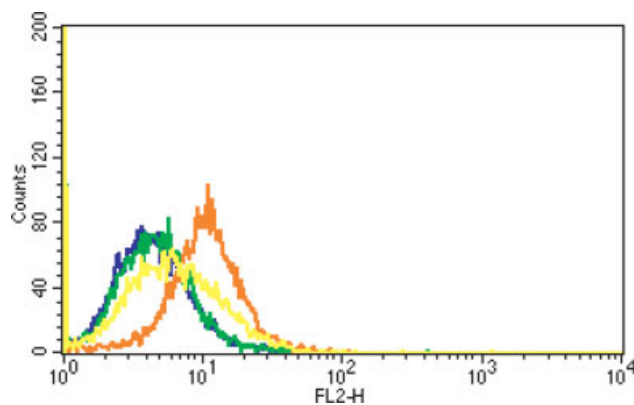


Figure 7. Flow cytometric analysis of cell uptake by Rho-labeled LMWP PR conjugate, Rho-labeled PR-LMWP and heparin mixture, Rho-labeled PR-LMWP, heparin and protamine mixture. Blank (blue), PR (green), Rho-labeled LMWP-PR conjugate (orange), Rho-labeled PR-LMWP and heparin mixture (yellow), and Rho-labeled PR-LMWP, heparin and protamine mixture (purple). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

loading on the PR-CPT conjugates was only $\sim 8\%$ (w/w), suggesting that only $\sim 10\%$ of the activated carboxyethyl ester binding sites on the PR chains were occupied by CPT molecules. This phenomenon may be accounted for by the steric hindrance that is imparted by the rather large CPT structure which could block additional drug loading. Other researchers have demonstrated that by introducing spacer arms even as small as glycine residues between the poly(glutamic acid) chain and the attached drug,²¹ drug loading can be significantly increased in the PR-based conjugates. Future investigation is therefore aimed towards further improvement of the drug loading of the PR-based drug carrier.

As expected, conjugation of CPT to PR resulted in certain polymer-induced beneficial effects, such as improved solubility and stability of the drug. Results showed that the hydrophilic character of PR improved the overall solubility of the hydrophobic CPT nearly sevenfold; from the initial 29–195 g/mL. In addition, linking of CPT to the PR polymer via the OH group at the C(20) position of CPT also protects the lactone ring of the drug molecule from hydrolysis, thereby preserving the therapeutic activity of the drug. It has been demonstrated by other investigators that when CPT is modified in a similar fashion by attaching the drug molecule to a poly (glutamic acid) polymer via the C(20)-OH group, it has displayed significantly enhanced stability and therapeutic efficacy *in vivo*.²¹

From *in vitro* release studies, a burst release ($\sim 20\%$) of CPT from the PR-drug conjugates was observed, followed by a nearly linear, sustained release of the drug over the period of more than 7 days. This release rate, which was governed primarily by the rate of hy-

drolysis of the ester bond between CPT and PR, could be readily manipulated by adopting different chemical linkages. It still remains unclear as to which factors may have contributed to the burst release of CPT at the initial stage, although nonspecific adsorption of free CPT molecules on the conjugates during their synthesis as well as unanticipated hydrolysis of CPT from the conjugates during storage may offer some speculations into this observation. Based on comparison of the molecular weight of the CPT-linked α -CD monomers (theoretical maximum: 3700 Da, practical average: 1430 Da) to the cut-off range of the dialysis tubing employed in the release studies (3.0 kDa), it seems likely that the released CPT was not the free form of drug, but rather the CPT-containing α -CD monomers resulting from dethreading of the PR-CPT conjugates. Identification of the presence of α -CD monomers in the release medium by NMR analysis will be conducted in future experiments to verify this possibility.

In vitro cytotoxicity results indicated that the ID₅₀ of the PR-CPT conjugates in A2780 cells was more than 200-fold higher than that for free CPT (1.41 μ M versus 0.006 μ M). This finding was expected, as the small and hydrophobic nature of CPT allows for cellular uptake to readily occur by passive diffusion. In contrast the membrane-impermeable, macromolecular PR-CPT conjugates could not be internalized by the cells to induce the cytotoxic effects of CPT. Interestingly but as anticipated, the LMWP-PR-CPT conjugates exhibited a 10-fold reduction in the ID₅₀ value when compared to the PR-CPT conjugates without LMWP (0.13M versus 1.41M). This increased cytotoxicity occurred probably because, even though the LMWP-PR-CPT conjugates were water soluble macromolecular structures, they were nevertheless internalized by the cells due to the demonstrated transmembrane activity of LMWP.¹⁷

As previously discussed, carrier-based delivery systems of small drugs with a sustained release feature (e.g., liposomal delivery systems) may achieve tumor accumulation via the EPR effect, followed by local release of drug at the tumor interstitium for the extended antitumor effect. However, the build-up of the interstitial concentration of the released drugs may cause the drugs to diffuse outside the tumor and potentially create the cytotoxic effects to the normal tissues surrounding the tumor. To overcome this obstacle, the enhanced intracellular uptake of the carrier at the tumor may be needed. To this regard, we have developed a novel strategy by integrating prodrug features into the LMWP-mediated intracellular drug delivery. During incubation of the LMWP-PR-CPT conjugates with tumor cells, an *in vitro* process that can partially mimic tumor targeting by the EPR effect, heparin was used to electrostatically mask the transmembrane activity of LMWP on the conjugates to yield the prodrug feature. Our

results showed that the addition of heparin was able to inhibit internalization of the LMWP-PR-CPT conjugates, although it appears that some translocation did occur. One hypothesis was that an insufficient amount of heparin was added to the LMWP-PR-CPT conjugates, rendering its inhibition on LMWP incomplete. Another possible explanation may be competition of cell surface heparin sulfate moieties with the administered heparin inhibitor. It has previously been demonstrated that negatively charged cell surface proteoglycans are required for PTD transduction. However, these exact mechanisms remain unconfirmed. Nevertheless, this prodrug function mediated by heparin to prevent internalization can inhibit the CPT-induced cytotoxic effects, and was clearly demonstrated by both confocal and flow cytometry results. Therefore, following hypothetical tumor accumulation of the macromolecular prodrug conjugates by EPR targeting, the LMWP-mediated cell internalization can be successfully restored by the addition of protamine to the heparin-inhibited drug conjugates. With this approach, this novel PR-based drug delivery system can be passively targeted to the tumor interstitium, but also allows for internalization of the complete polymer-drug complex as opposed to requiring hydrolysis and subsequent drug diffusion into tumor cells.

In conclusion, the *in vitro* feasibility of this novel PR-based CPT delivery system was demonstrated. Extensive animal studies are currently in progress in our laboratory, with the aims at further validating this novel CPT delivery strategy. It should be pointed out that should *in vivo* tumor targeting and therapy prove successful, this PR-based strategy could evolve as a generic system for the delivery of various small antitumor agents that possess poor solubility and reaction selectivity.

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References

- Wall ME, Wani MC, Cook CE, Palmar KH, McPhail AT, Sim GA. Plant tumor agent. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibition from *Camptotheca acuminata*. *J Am Chem Soc* 1966;88:3888-3890.
- Kingsbery WD, Boehm JC, Jakas DR, Holden KG, Hecht SM, Gallagher MJ, Caranfa FL, McCabe FL, Faucette LF, Johnson RK, Hertzberg RP. Synthesis of water soluble (aminoalkyl) camptothecin analogues: Inhibition of topoisomerase I and antitumor activity. *J Med Chem* 1991;31:98-107.
- Takimoto CH, Arbuck SG. Topoisomerase I Targeting Agent: The Camptothecins. Philadelphia: Lippincott Williams & Wilkins; 2001. p 579-646.
- Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumor-tropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 1986;46(12, Part 1):6387-6392.
- Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. *Am J Pathol* 1988;133:95-109.
- Minko T, Kopeckova P, Kopecek J. Comparison of the anticancer effect of free and HPMA copolymer-bound adriamycin in human ovarian carcinoma cells. *Pharm Res* 1999;16:986-996.
- Yui N. New world of micromachines based on supramolecular assemblies. *Micromachine* 1999;29:3-4.
- Ichi T, Watanabe J, Ooya T, Yui N. Controllable erosion time and profile in poly(ethylene glycol) hydrogels by supramolecular structure of hydrolyzable polyrotaxane. *Biomacromolecules* 2001;2:204-210.
- Ooya T, Yui N. Polyrotaxanes: Synthesis, structure, and potential in drug delivery. *Crit Rev Ther Drug Carrier Syst* 1999;16:289-330.
- Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. In vivo protein transduction: Delivery of a biologically active protein into the mouse. *Science* 1999;285:1569-1572.
- Elliott G, O'Hare P. Intercellular trafficking and protein delivery by a herpes virus structural protein. *Cell* 1997;88:223-233.
- Joliot A, Pernelle C, Deagostini-Bazin H, Prochiantz A. Antennapedia homeobox peptide regulates neural morphogenesis. *Proc Natl Acad Sci USA* 1991;88:1864-1868.
- Suzuki T, Futaki S, Niwa M, Tanaka S, Ueda K, Sugiura Y. Possible existence of common internalization mechanisms among arginine-rich peptides. *J Biol Chem* 2002;277:2437-2443.
- Chang LC, Lee HF, Yang Z, Yang VC. Low molecular weight protamine (LMWP) as nontoxic heparin/low molecular weight heparin antidote (I): Preparation and characterization. *AAPS Pharm Sci* 2001;3:E17.
- Chang LC, Liang JF, Lee HF, Lee LM, Yang VC. Low molecular weight protamine (LMWP) as nontoxic heparin/low molecular weight heparin antidote (II): In vitro evaluation of efficacy and toxicity. *AAPS PharmSci* 2001;3:E18.
- Lee LM, Chang LC, Wroblewski S, Wakefield TW, Yang VC. Low molecular weight protamine as nontoxic heparin/low molecular weight heparin antidote (III): Preliminary in vivo evaluation of efficacy and toxicity using a canine model. *AAPS PharmSci* 2001;3:E19.
- Park YJ, Chang LC, Liang JF, Moon C, Chung CP, Yang VC. Nontoxic membrane translocation peptide from protamine, low molecular weight protamine (LMWP), for enhanced intracellular protein delivery: In vitro and in vivo study. *FASEB J* 2005;19:1555-1557.
- Dietz GP, Bahr M. Delivery of bioactive molecules into the cell: The Trojan horse approach. *Mol Cell Neurosci* 2004;27:85-131.
- Harada A, Kamachi M. Complex formation between poly(ethylene glycol) and α -cyclodextrin. *Macromolecules* 1990;23:2821-2823.
- Han G, Tamaki M, Hruby VJ. Fast, efficient and selective deprotection of the *tert*-butoxycarbonyl (Boc) group using HCl/dioxane (4 m). *J Pept Res* 2001;58:338-341.
- Singer JW, Bhatt R, Tulinsky J, Buhler KR, Heasley E, Klein P, de Vries P. Water-soluble poly(L-glutamic acid)-Gly-camptothecin conjugates enhance camptothecin stability and efficacy in vivo. *J Control Release* 2001;74:243-247.