Novel Polymeric Prodrug with Multivalent Components for Cancer Therapy

Jayant J. Khandare, Pooja Chandna, Yang Wang, Vitaly P. Pozharov, and Tamara Minko
Department of Pharmaceutics, Rutgers, The State University of New Jersey, Piscataway, New Jersey
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ABSTRACT
We designed, synthesized, and evaluated in vitro and in vivo a novel targeted anticancer polymeric prodrug containing multiple copies of tumor targeting moiety [synthetic luteinizing hormone-releasing hormone (LHRH) peptide, analog of LHRH] and anticancer drug (camptothecin). One, two, or three molecules of the targeting peptide and anticancer drug were covalently conjugated with bis(2-carboxyethyl) polyethylene glycol polymer using citric acid as a multivalent spacer. We showed that LHRH peptide was bound to extracellular receptors and localized in plasma membrane of cancer cells. The designed tumor-targeted prodrug increased the solubility of anticancer drug and offered cytoplasmic and/or nuclear delivery of drug to cancer cells expressing LHRH receptors. The multicomponent prodrug containing three copies of the targeting peptide and drug was almost 100 times more cytotoxic and substantially had enhanced antitumor activity compared with the analogous nontargeted prodrug and prodrugs containing one or two copies of active components.

Many anticancer drugs used in chemotherapy require modifications to increase solubility, decrease adverse side effects, limit nonspecific activity, increase circulation time, modify biodistribution, and so on. Various drug delivery systems (DDS) have been developed to provide these modifications (Lasic and Papahadjopoulos, 1995; Zhang et al., 1996; Uhrich et al., 1999; Allen et al., 2000; Kataoka et al., 2001; Langer, 2001; Discher and Eisenberg, 2002; Ihre et al., 2002; Torchilin, 2002). Established DDS primarily consist of polymeric conjugations or liposomal formulations (Rihova et al., 2001; Minko et al., 2002; Greenwald et al., 2003a; Allen and Cullis, 2004; Yoo and Park, 2004). Recent innovations in dendritic polymers have been considered as the most promising “nanocarriers” to deliver high payload of drug and other therapeutic components (Kono et al., 1999; Malik et al., 2000; Khandare et al., 2005). However, these novel polymers and their conjugates await proof of clinical safety and efficacy.

Targeted anticancer DDS offer further drug modifications and provide for the so-called “advanced targeted prodrug approaches” (Minko, 2005). Because of such conjugation, or encapsulation into a carrier, therapeutic compounds are said to form a “prodrug,” which is inactive during the delivery to the site of action and is converted into an active drug at the targeted organ, tissues, or cells. Luteinizing hormone-releasing hormone (LHRH) peptide was used as a targeting moiety (ligand) to LHRH receptors that are overexpressed in the plasma membrane of several types of cancer cells and are not expressed and detectable in normal visceral organs (Furui et al., 2002; Dharap and Minko, 2003; Dharap et al., 2003, 2005; Minko et al., 2004). LHRH-containing prodrugs preferentially accumulated in tumors, limited adverse side effects on healthy organs, and substantially enhanced tumor toxicity of an anticancer drug.

Polyethylene glycol (PEG) polymer is widely used as a carrier for DDS (Greenwald, 2001). PEG is a water-soluble nonionic polymer approved by the Food and Drug Administration for pharmaceutical applications. Because of its nontoxic character, it is widely used in many biochemical, cosmetic, pharmaceutical, and industrial applications. It is also important that PEG polymers show low-antigen activity and in most cases decrease the antigenicity of active ingredients conjugated to them (Calici et al., 2001). Successful bioconjugation depends on the chemical nature, structure, molecular weight, steric hindrance, and reactivity of the biomolecule, as well as that of the polymer. In most prodrug conjugations, a variety of spacers can be incorporated be-

ABBREVIATIONS:
DDS, drug delivery system(s); LHRH, luteinizing hormone-releasing hormone; PEG, polyethylene glycol; CPT, camptothecin; CA, citric acid; DMAP, 4-(methylamino)pyridine; FITC, fluorescein isothiocyanate; EPR, enhanced permeability and retention; DMF, dimethylformamide; DCM, dichloromethane; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline.
tween polymer and biomolecules, because they can offer a chemical flexibility and can be hydrolyzed to release the bioactive component (Kopecek et al., 2000, 2001). The suitability of a spacer molecule depends on its linearity, molecular weight, end functionality, charge, and reactivity. Current bioconjugation methodologies are limited to only one drug molecule per polymeric chain. On the other hand, polymers can deliver multiple drug molecules simultaneously to the cancer cells, thereby accumulating higher concentration of a drug and consequently increasing the efficacy of cancer treatment. In the present article, we report the design, synthesis, and evaluation of a novel water-soluble polymeric targeted multivalent anticancer prodrug, which is able to simultaneously deliver several copies of anticancer drugs specifically to tumor. The described system consists of up to three copies of each targeting moiety (synthetic LHRH peptide, analog of LHRH) and anticancer drug (camptothecin (CPT)) conjugated to bis-carboxyl functional PEG polymer via multivalent spacer. However, the design of the developed delivery system is not limited to only three copies of active ingredients per one molecule of PEG carrier. The proposed novel synthetic approach based on the use of citric acid (CA) as a multivalent spacer allows conjugation of substantially higher number of copies of active ingredients and may be utilized for the delivery of different drugs and imaging agents, as well as other LHRH-targeting moieties.

The number of molecules of conjugated substances per one molecule of PEG is limited mainly by their solubility, not by the design, synthesis of the whole system, steric hindrance of components, and so on. Relative simplicity of the proposed novel synthetic procedure and the ability to use multiple copies of drugs, peptides, targeting moieties, imaging agents, or other active components make the proposed system unique and open the door for a new family of multivalent anticancer drugs and other therapeutic and diagnostic agents.

Materials and Methods

Cell Line. The human ovarian carcinoma A2780 cell line was obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Cells were grown at 37°C in a humidified atmosphere of 5% CO2 (v/v) in air. All of the experiments were performed on cells in the exponential growth phase.

Synthesis of α,ω-Bis-(2-Carboxyethyl) PEG-CPT Conjugates. CPT, N,N-di-isopropyl-ethylamine, and 4-(methylamino)pyridine (DMAP) were obtained from Sigma Chemical Co., Atlanta, GA; bis(2-carboxyethyl) PEG molecular mass of ~3000 Da polymer and fluorescein isothiocyanate (FITC) were obtained from Fluka (Allentown, PA). CPT is a quinoline-based indole alkaloid and is a close chemical entity of aminocamptothecin, CPT-11 (generic name, irinotecan), DX-8951f, and topotecan, found in the bark of the Chinese camptotheca tree and the Asian nothapodytes tree. Rhodamine red succinimidyl ester and Hoechst 33258 dye were purchased from Invitrogen-Molecular Probes (Carlsbad, CA). The selection of molecular weight of PEG polymer was based on the following consideration. On one hand, an increase in the molecular weight of the carrier improves drug pharmacokinetics and enhances its accumulation in the tumor. On the other hand, an increase in molecular weight of polymer substantially limits drug bioavailability and cytotoxicity. Based on our previous studies (Minks et al., 2000a,b, 2002, 2004; Dharap et al., 2005), we selected PEG polymer with a molecular mass of 3000 Da, which shows only moderate decrease in bioavailability and cytotoxicity of CPT while improving drug pharmacokinetics. Such relatively low molecular weight polymer does not provide for effective passive tumor targeting by the enhanced permeability and retention (EPR) effect. However, the inclusion of LHRH peptide as a targeting moiety in the delivery system ensures very effective tumor targeting and retention of CPT-PEG-LHRH conjugates (Dharap et al., 2005).

α,ω-Bis-(2-Carboxyethyl) PEG-CAT Conjugates (Compounds 3 in Fig. 2B). α,ω-Bis-(2-carboxyethyl) PEG3000-CA conjugate was synthesized using a one-step procedure (Fig. 2). CA (compound 2 in Fig. 2B; CAS no. 77-92-8, 2-hydroxy-1,2,3-propanetricarboxylic acid) contains one hydroxyl and three carboxyl functional reactive groups for chemical conjugation. In brief, bis-(2-carboxyethyl) PEG (compound 1 in Fig. 2, 100 mg, 0.033 mM) and 2 mol of CA (12.8 mg, 0.033 mM) were dissolved in 5 ml of anhydrous dimethylformamide (DMF) and 20 ml of anhydrous dichloromethane (DCM). N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide HCl (EDC-HCl, 13 mg, 0.0678 mM) was added to this solution as a coupling agent, and DMAP (4.03 mg, 0.032 mM) was used as a catalyst. The reaction solution was stirred continuously for 24 h at room temperature. The carboxidimide urea formed during the reaction was removed by filtration. The unreacted CA and EDC-HCl were removed by dialysis using Spectra/Por membrane (molecular mass cutoff, ~2000 Da) in DMSO as a solvent. Further purification of the α,ω-bis-PEG-CA conjugate was carried out using size-exclusion SephadeX G10 columns. The conjugate was dried under the vacuum at room temperature.

α,ω-Bis-(2-Carboxyethyl) PEG-Citrate-CPT Conjugate (Compounds 5, 6, 7 in Fig. 2C). α,ω-Bis-(2-carboxyethyl) PEG3000-CA conjugate (compound 3 in Fig. 2B) (molecular mass, ~3382.24 Da, 50 mg, 0.0147 mM) and CPT (compound 4 in Fig. 2C) (5.2 mg, 0.0147 mM) were dissolved in 5 ml of anhydrous dimethyl sulfoxide (DMSO) and 10 ml of anhydrous DCM. The reaction mixture was allowed to stir for 30 min. EDC-HCl (3 mg, 0.0156 mM) was added to the above solution as a condensing agent, and DMAP (2.0 mg, 0.016 mM) was used as a catalyst. The reaction was stirred continuously for 24 h at room temperature. The carboxidimide urea formed during the reaction was removed by filtration. The unreacted CPT and EDC-HCl were removed by dialysis using Spectra/Por dialysis membrane (molecular mass cutoff, ~2000 Da) in DMSO as a solvent. The conjugate was dried under vacuum at room temperature. In addition, polymer conjugates with two and three copies of CPT were prepared by using two and three molar ratios of CPT and EDC-HCl, respectively. 1H NMR spectra of α,ω-bis-(2-carboxyethyl) PEG-CPT conjugates were recorded on Varian 400-MHz spectrophotometer using DMSO-d6 as a solvent. 1) CPT protons correspond to δ 0.9 CH3 (t), δ 1.9 CH2 (m), δ 5.35 CH3 (s), δ 5.5 CH2 (s), 6.7 CH (s), δ 7.0 CH (s), 7.65 to 7.8 to 8.0 2H, m-CH, δ 8.2-OO-CH, O-CH (d), and 2) bis-(2-carboxyethyl) PEG protons correspond from δ 3.2 to 3.8 broad peaks (Fig. 3A).

α,ω-Bis-(2-Carboxyethyl) PEG-Citrate-CPT-LHRH Conjugate (Compounds 5a, 6a, 7a in Fig. 2D). α,ω-Bis-(2-carboxyethyl) PEG3000-CA-CA-1CPT conjugate (molecular mass, ~3729 Da, 50 mg, 0.0134 mM) and peptide LHRH-NH2 (18 mg, 0.013 mM) were dissolved in 5 ml of anhydrous dimethylformamide (DMF) and 20 ml of anhydrous dichloromethane (DCM). N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide HCl (EDC-HCl, 13 mg, 0.0678 mM) was added to this solution as a coupling agent, and DMAP (4.03 mg, 0.032 mM) was used as a catalyst. The reaction solution was stirred continuously for 24 h at room temperature. The carboxidimide urea formed during the reaction was removed by filtration. The unreacted CPT and EDC-HCl were removed by dialysis using Spectra/Por dialysis membrane (molecular mass cutoff, ~2000 Da) in DMSO as a solvent. The conjugate was dried under vacuum at room temperature. In addition, polymer conjugates with two and three copies of CPT were prepared by using two and three molar ratios of CPT and EDC-HCl, respectively. 1H NMR spectra of α,ω-bis-(2-carboxyethyl) PEG-CPT conjugates were recorded on Varian 400-MHz spectrophotometer using DMSO-d6 as a solvent. 1) CPT protons correspond to δ 0.9 CH3 (t), δ 1.9 CH2 (m), δ 5.35 CH3 (s), δ 5.5 CH2 (s), 6.7 CH (s), δ 7.4 CH (s), 7.65 to 7.8 to 8.0 2H, m-CH, δ 8.2-OO-CH, O-CH (d), and 2) bis-(2-carboxyethyl) PEG protons correspond from δ 3.2 to 3.8 broad peaks (Fig. 3A).

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α,α-Bis(2-Carboxyethyl) PEG-FITC Conjugate. Hydroxyl groups of PEG were condensed with the carboxyl group of bis(2-carboxyethyl) PEG using EDC-HCl as coupling agent. In brief, bis-PEG (50 mg, 0.016 mM) and FITC (6.2 mg, 0.016 mM) were dissolved in 2 ml of anhydrous DMSO and 10 ml of anhydrous DCM. EDC-HCl (4 mg, 0.020 mM) was added as a condensing agent, and DMAP (2.0 mg, 0.016 mM) was used as a catalyst. The reaction was stirred for 24 h under subdued light. The reaction solution was filtered to remove the carboxidiimide salt. The conjugate was washed with excess acetone three times to remove free FITC, and the conjugate was precipitated in diethyl ether. Furthermore, bis-PEG-FITC conjugate was purified by dialysis using Spectra/Por dialysis membrane (molecular mass cutoff, ~2000 Da) in DMSO as a solvent. The conjugate was further purified by size-exclusion G10 Sephadex column chromatography.

LHRH-Rhodamine Red Succinimidyl Ester Labeling. Synthetic analog of LHRH peptide was synthesized according to our design (Dharap et al., 2003, 2005) by American Peptide Company, Inc. (Sunnyvale, CA). Rhodamine red succinimidyl ester was covalently conjugated, with LHRH peptide having NH2 at the terminal. In brief, LHRH (5.0 mg, 0.0037 mM) and LHRH-NH2 (3 mg, 0.0039 mM) were dissolved in 2.0 ml of anhydrous DMF, and 4.0 ml of N,N-di-isopropyl-ethylamine was added to adjust alkaline pH and maintain amine group in nonprotonated form. The reaction was stirred for 2 h under subdued light. The conjugate was washed three times using acetone to remove free rhodamine red succinimidyl ester. The conjugate was further purified by size-exclusion G10 Sephadex column chromatography to remove nonconjugated LHRH and rhodamine red ester.

UV Analysis of LHRH and LHRH-Containing Conjugates. One milligram of standard LHRH-NH2, 3× CPT-PEG (no LHRH), 3× CPT-PEG-1× LHRH, 3× CPT-PEG-2× LHRH, and 3× CPT-PEG-3× LHRH conjugates were dissolved in 1 ml of deionized water, and UV spectra were recorded from 250 to 450 nm (Fig. 3C).

Fluorescence Measurements. The concentration of CPT was estimated using fluorescence spectrophotometer (excitation 360 nm and emission 465 nm) with gain of 40 and number of flashes = 3. Varying concentrations of CPT were prepared using DMSO as a solvent. The fluorescence intensity was measured for each lowered dilution, and the standard plot was drawn for concentration in milligram per milliliter. Amount of CPT in bis(2-carboxyethyl) PEG-CA-CPT conjugates with one, two, and three copies of CPT was estimated using CPT standard plot.

Molecular Modeling. Energy minima and molecular dynamics for conformational structure were studied with bis-PEG-CPT conjugates with and without CA spacer. The bis-PEG polymer (molecular mass, ~3000 Da) linked to CA and/or CPT molecule structures were built using ChemDraw 9.0 Pro (CambridgeSoft Corp, Cambridge, MA). Distance between the first and last carbon atom was measured for molecularly dynamic structure with CA or no CA having different number of copies of CPT molecules using RasTop Molecular Visualization Software (Philippe Valadon, San Diego, CA) (Fig. 4, A and B). The bioconjugate with PEG was built to represent seven ethylene repeat (–CH2–O–CH2–) units. The settings for energy minima and molecular dynamics were order 1,000-step interval, 2.0 fs; frame interval, 10 fs; terminate after 10,000 steps; heating/cooling rate, 1,000 kcal/atom/ps; and target temperature, 300 K.

In Vitro Cellular Entry of Conjugates. A2780 human ovarian cancer cells were seeded in a six-well culture plate (1×104 cells/well) and made up to 2 ml of final volume supplemented with 10% fetal bovine serum and 1% penicillin. The cells were incubated for 24 h at 37°C with CPT, 3× CPT-PEG, and 3× CPT-PEG-3× LHRH conjugate. Conjugates were dissolved in phosphate-buffered saline (PBS) buffer (pH 7.4), whereas free CPT was first dissolved in 5% DMP and then added to PBS buffer. One hundred microliters of supernatant was removed at intervals of 0, 5, 10, 15, 30, 45, and 60 min and at intervals of 3, 6, and 24 h. Equal volume of media was replaced to each well. The supernatant was centrifuged at ~12,000g for 5 min using Eppendorf centrifuge 5415D (Brinkmann Instruments, New York, NY) to remove the cellular debris. Cellular uptake for conjugates was measured by estimating fluorescence (excitation 360 nm and emission 465 nm) using calibration curve of standard CPT.

In Vitro Cytotoxicity. The cytotoxicity of CPT conjugates and free CPT was assessed by using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (Maeda, 2001; Dharap and Minko, 2003; Fang et al., 2003; Greish et al., 2003; Minko et al., 2003).

In Vivo Antitumor Activity. Previously developed mouse model of human ovarian carcinoma xenografts was used (Minko et al., 2000a,b; Dharap et al., 2005). In brief, 2×106 A2780 human ovarian carcinoma cells were transplanted s.c. into the flanks of female athymic nu/nu mice. When the tumors reached a size of approximately 1 cm3 (~15–20 days after transplantation), mice were separately treated i.p. with saline (control), CPT, and 1× CPT-PEG, 2× CPT-PEG, 3× CPT-PEG, 1× CPT-PEG-1× LHRH, 3× CPT-PEG, 2× CPT-PEG-3× LHRH conjugates. The dose of all of the substances (10 mg/kg for the single injection) corresponded to the maximal tolerated dose of CPT. Equivalent CPT concentrations were 3.8, 5.6, and 5.6 mg/kg for conjugates containing one, two, and three copies of CPT, respectively. Maximal tolerated dose was estimated in separate experiments based on animal weight changes after the injection of increasing doses of the drugs as described previously (Minko et al., 2000a,b; Dharap et al., 2005). Tumor size was measured at 6, 12, 18, 24, 36, 48, 72, and 96 h after the treatment. Changes in tumor size were used as an overall mark of antitumor activity.

Statistics. All of the in vitro and in vivo experiments were performed in quadruplicate. The results are expressed as mean ± S.D. from four to eight independent measurements. Statistical analysis was performed as a one-way analysis of variance, and comparisons among groups were performed by independent sample t test.

Results

Cellular Localization of LHRH Peptide and PEG Polymer after Incubation with Cancer Cells

Previously, we (Dharap and Minko, 2003; Dharap et al., 2003, 2005; Minko et al., 2004) and others (Furui et al., 2002) have reported that receptors for LHRH are overexpressed in many types of cancer cells, including ovarian, breast, and prostate carcinoma, and are not expressed in healthy visceral organs. Although LHRH receptors were slightly expressed in healthy ovarian, breast, and prostate tissues, the expression of these receptors in tumors was substantially higher compared with normal tissues from the same organ of the same patient (Dharap et al., 2005). This allows us to use LHRH peptide as a targeting moiety to deliver anticancer drugs to tumors that overexpress LHRH receptors. To show that LHRH peptide and PEG polymer can be used as a targeting moiety and a delivery vehicle, respectively, we studied intracellular distribution of LHRH peptide and PEG after incubation with A2780 human ovarian carcinoma cells expressing LHRH receptors (Dharap and Minko, 2003; Dharap et al., 2003, 2005; Minko et al., 2004). The distribution study of labeled LHRH and PEG (Fig. 1) showed that rhodamine-labeled LHRH peptide accumulated predominantly in the plasma membrane and part of the cellular cytoplasm adjacent to the outer cellular membrane. In contrast, FITC-labeled PEG polymer was almost equally distributed in the cellular cytoplasm and nuclei.
Synthesis and Characterization of Targeted Anticancer Prodrug with Several Copies of LHRH Peptide and CPT

Our ultimate goal was to synthesize a complex targeted anticancer prodrug with multiple copies of targeting moiety (synthetic analog of LHRH peptide) and anticancer drug (CPT) per one molecule of PEG (Fig. 2A). In this study we used one, two, or three copies of each active component to show the feasibility of the proposed approach. However, conjugates containing one, two, and three copies of CPT substantially enhanced aqueous solubility of the drug. This solubility was 15.0, 10.0, and 6.5 mg/ml for conjugates with one, two, and three CPT copies, respectively. In contrast, free CPT is practically insoluble in aqueous solutions. The details of the synthesis are summarized below.

α,ω-Bis(2-Carboxyethyl) PEG-CA Conjugate. The PEG polymer reported here was α,ω-bis(2-carboxyethyl) PEG (molecular mass, ~3000 Da) and was a diacid with the molecular formula HOOCCH₂CH₂CONH(CH₂)₅CH₁₀C-H₁₈NCOCH₂CH₂COOH. Two moles of CA were coupled (i.e., hydroxyl group of CA), with 1 mol of carboxyl-terminated bis-PEG using EDC-HCl as a coupling agent to form tricarboxylate groups at both terminals (Fig. 2B). Multifunctional CA was used as a spacer to decrease the steric hindrance and increase the reactivity of PEG-CA conjugate with CPT. The resulting bis-PEG-CA conjugate was purified using molecular mass cutoff dialysis membrane (molecular mass, ~2000 Da) and size-exclusion G10 Sephadex column. The bis-PEG-CA conjugate contained a total number of six carboxylate groups for further conjugation with multiple copies of CPT-OH and LHRH-NH₂.

α,ω-Bis(2-Carboxyethyl) PEG-CA-CPT Conjugate. Hydroxyl group at the position 20 of CPT was covalently coupled with carboxyl groups of bis-PEG-CA to form a degradable ester bond. One, two, and three copies of CPT per bis-PEG-CA conjugate were obtained by reacting 1, 2, and 3 mol of CPT with 1 mol of polymer, respectively (Fig. 2C). Likewise, the molar ratio of EDC-HCl and DMAP were varied. Polymer conjugate with one, two, and three copies of CPT showed substantially higher aqueous solubility compared with free CPT, which is not soluble in aqueous solutions. The formation of CPT-bis-PEG conjugates was confirmed by proton and ¹³C nuclear magnetic resonance (¹H NMR and ¹³C NMR). The aromatic peaks in Fig. 3A indicate the presence of CPT molecules along with broad peaks for bis-PEG, whereas ¹³C NMR confirmed the formation of ester bond between CPT and PEG-CA (data not shown). In addition, ¹³C NMR shows the peaks for free acidic groups in the conjugate. The amount of CPT per PEG in conjugates using proton integration method was found to be 30%, 38.12%, and 55.85% (w/w) for one, two, and three copies of CPT, respectively. The theoretical ratio for the conjugation of CPT for one, two, and three copies of CPT is 11%, 22%, and 33% (w/w), respectively. The increased conjugation ratio in reactions may be because of the presence of multiple carboxyl groups for conjugation of drug with polymer. The integration ratios for PEPEG peaks were decreased with increased number of copies of CPT. MALDI/TOF (PE Biosystems Voyager System 6080) showed increased molecular weight for CPT-PEG conjugate. Furthermore, the concentration of CPT in each conjugate was estimated using fluorescence spectroscopy at excitation of 360 nm and emission of 465 nm. We selected fluorescence spectroscopy for the measurement of CPT based on the following considerations. First, CPT has a wide range of UV absorbance wavelengths, and the detection limit of the drug absorbance is relatively high. In contrast, the fluorescence measurements are more sensitive, even with low concentrations of CPT using emission of ~360 nm and excitation of ~465 nm. Second, we found that PEG itself does not interfere with the fluorescence measurements of CPT in the conjugates and is not detected in the emission and excitation range of wavelength to that of CPT up to concentrations of 1.0 mg/ml. The detection limit of CPT by fluorescence spectroscopy was 0.0007 mM concentration.

α,ω-Bis(2-Carboxyethyl) PEG-CA-CPT-LHRH Conjugate. LHRH analog, Lys₆-des-Gly₁₀-Pro₉-ethylamide (Gln-His-Trp-Ser-Tyr-d-Lys-Leu-Arg-Pro-NH₂-Et), having a reactive amino group only on the side chain of the lysine at position 6 was reacted with free carboxyl groups in α,ω-bis(2-carboxyethyl) PEG-CA-CPT conjugate to form a nondegradable amide bond (Fig. 2D). The amount of LHRH to obtain one, two, and three copies per bis-PEG-CA-CPT conjugate was manipulated on a molar basis. Likewise, the molar ratios of EDC-HCl and DMAP were varied. The concentration of CPT in the each conjugate was estimated by fluorescence spectroscopy at excitation of 360 nm and emission of 465 nm. MALDI/TOF showed increased molecular weight for LHRH-PEG-CA-CPT conjugates (Fig. 3B).

Molecular Modeling. Conformational structures for conjugates 3, 5, 6, and 7 (Fig. 4), with one, two, and three copies of CPT, were explored using molecular modeling studies. Because of high molecular weight constraints, the three-dimensional conformation of polymers along with conjugated...
biomolecules is not being elucidated. It was thought worthwhile to build a molecular conformation and evaluate energy minima, stable conformations, and molecular dynamics for synthesized conjugates (Fig. 4). The minima represented the energies (kcal) indicating degree of conformational freedom for seven repeating units of bis-PEG polymeric chain along with CA as a spacer. In addition, molecular dynamic conformations were established for conjugates with one and three copies of CPT molecules. Figure 4A shows the molecular dynamic conformation for the bis-PEG-CA conjugate containing three copies of CPT, whereas Fig. 4B represents distance and adjacent molecular alignments for the same conjugate. Distance between the first and last carbon atom for molecular dynamic structure of free CPT was 8.887 and 15.83 Å for bis-PEG-CA-1CPT. In addition, distance for bis-PEG-CA with three CPT molecules was observed to be 13.74 Å, whereas it was 12.23 Å with energy minima. On the other hand, bis-PEG-1/H9251 CPT conjugate without CA spacer exhibits distance of 6.05 Å. The results indicate that the longer PEG polymeric chains do collapse in the presence of CA spacer. It was surprising to note that the distance decreased between the carbon molecules in the presence of three CPT molecules over to one CPT molecule in bioconjugate.

UV Analysis of LHRH and LHRH-Containing Conjugates. To show the presence of targeting LHRH-NH₂ peptide in the conjugate, UV spectra were recorded for LHRH-NH₂

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**Fig. 2.** Synthesis of targeted multivalent anticancer prodrug. The system (a) contains bis-PEG polymer as a carrier; one, two, or three copies of LHRH peptide as a targeting moiety; and one, two, or three copies of CPT as an anticancer drug. b, α,ω-bis-PEG₃₀₀₀₀-CA conjugate (3) was synthesized using one-step procedure by conjugation of 1 M bis(2-carboxyethyl) PEG (1) with 2 M CA [CAS no. 77-92-9, 2-hydroxy-1,2,3-propanetricarboxylic acid] (2). The bis-PEG-CA conjugate (3) was coupled with 1, 2, and 3 mol of CPT (4) to obtain α,ω-bis(2-carboxyethyl) PEG-CA-CPT conjugates (c, 5, 6, and 7). Furthermore, 1, 2, and 3 mol of LHRH (8) were conjugated with c, 5, 6, and 7 to obtain α,ω-bis(2-carboxyethyl) PEG-CA-CPT-LHRH conjugates (d, 5a, 6a, and 7a) having one, two, and three copies of CPT (4) and LHRH (8).
peptide alone, 3×CPT-PEG (no LHRH), 3×CPT-PEG-1×LHRH, 3×CPT-PEG-2×LHRH, and 3×CPT-PEG-3×LHRH conjugates (Fig. 3C). The increase in UV absorbance at ~280 nm for conjugates with one, two, or three copies of LHRH peptide indicates higher amount of peptide incorporation in the conjugate.

**In Vitro Cellular Uptake of 3×CPT-PEG and 3×CPT-PEG-3×LHRH Conjugates.** To show internalization of conjugates by ovarian cancer cells, we examined the cellular uptake of CPT, 3×CPT-PEG, and 3×CPT-PEG-3×LHRH conjugates using A2780 human ovarian carcinoma cells. As seen in Fig. 3D, almost 20% of the applied bis-PEG conjugate with three copies of CPT entered the cell within 5 min, indicating rapid internalization of the drug. The cellular entry was further increased to 26% within 20 min and reached a plateau. In contrast, free CPT was internalized by the cells significantly slower when compared with 3×CPT-PEG conjugate. Only approximately 12% of the applied dose of CPT entered the cells within 30 min. Moreover, it should be noted that, because of very low aqueous solubility of CPT, the drug was first dissolved in 5% DMF and further added to PBS buffer having pH 7.4. Addition of three copies of target-
ing moiety (synthetic analog of LHRH peptide) to the above polymer substantially enhances internalization of the conjugate, whereas final intracellular concentration of 3×CPT-PEG-3×LHRH conjugate was almost three times higher compared with the concentration of conjugate without LHRH. Therefore, conjugation of CPT to PEG substantially enhanced solubility and cellular availability of water-non-soluble drug CPT, whereas LHRH peptide provided further increase in bioavailability of CPT.

**Increasing of LHRH and CPT Copies per One Molecule of PEG Polymer Enhances Cytotoxicity and Antitumor Activity of Whole Prodrug**

**In Vitro Cytotoxicity.** Conjugation of CPT with PEG polymer resulted in high molecular weight complex and a decrease in its toxicity (increased IC₅₀ dose) when compared with CPT alone (Fig. 5). The increase in number of CPT molecules in the conjugate from one to two or three per one molecule of PEG led to the increase in the toxicity of the whole system of 1.9 and 3.1 times, respectively. An addition of one molecule of LHRH (targeting moiety/penetration enhancer) led to the increase in the toxicity of CPT-PEG conjugate with one, two, or three copies of the drug of 5.3, 8.87, and 12.9 times, respectively, compared with 1×CPT-PEG conjugate. An increase in the number of LHRH copies consequently led to the further increase in the anticancer activity of conjugates. As a result, targeted 3×CPT-PEG-2×LHRH and 3×CPT-PEG-3×LHRH conjugates were 30 and 77 times more toxic against human ovarian carcinoma cells compared with nontargeted 1×CPT-PEG conjugate. Preliminary studies showed that simple mixing without conjugation of PEG with CPT alone or CPT and LHRH does not attenuate CPT toxicity in tumor cells.

**In Vivo Antitumor Activity.** We analyzed the antitumor activity of the developed conjugates containing different copies of active ingredients on mice bearing xenografts of human ovarian tumor. Although, in general, in vivo data corroborate in vitro toxicity experiments, one substantial difference between these series was revealed. In contrast to the in vitro data where free CPT was more toxic than most conjugates (except conjugates containing three copies of CPT and two or three copies of LHRH), even nontargeted polymeric CPT-PEG conjugates with one to three copies of CPT were substantially more effective in terms of suppressing tumor growth (compare curves 2–4 with curve 1 on Fig. 6 with bars 1–4 on Fig. 5). This observation supports our previous findings related to “passive tumor targeting” by high molecular weight polymeric drugs because of the EPR effect (Minko et al., 2000a,b, 2004; Minko, 2005). Further enhancement of

**Fig. 6.** Inclusion of several copies of anticancer drug (CPT) and targeting moiety (synthetic analog of LHRH peptide) substantially enhances antitumor activity of prodrug toward xenografts of human ovarian tumor. A2780 human ovarian carcinoma cells were transplanted s.c. into the flanks of female athymic nu/nu mice. When the tumors reached a size of approximately 1 cm³ (15–20 days after transplantation), mice were treated once with each drug conjugate. The dose of all of the substances (10 mg/kg for the single injection) corresponds to the maximal tolerated dose of CPT. Equivalent CPT concentrations were 3, 3.8, and 5.6 mg/kg for conjugates containing one, two, and three copies of CPT, respectively. Tumor size was measured at 6, 12, 18, 24, 48, 72, and 96 h after the treatment. Mean ± S.D. are shown. *, P < 0.05 compared with control. †, P < 0.05 compared with free CPT. ‡, P < 0.05 compared with 1×CPT-PEG.

**Discussion**

The use of synthetic analog of natural LHRH peptide as a targeting moiety was based on the following main considerations. First, the receptor for this peptide was overexpressed in several cancer cells, including ovarian, endometrial, breast, and prostate cancers (Furuji et al., 2002; Dharap and Minko, 2003; Dharap et al., 2003, 2005; Minko et al., 2004). Second, although these receptors are expressed in healthy ovarian, breast, and prostate tissues, expression of LHRH receptors in corresponding tumors is substantially higher compared with normal tissues from the same patient (Dharap et al., 2005). Third, conjugation of LHRH peptide(s) to high molecular weight delivery system should enhance its uptake by cancer cells that overexpress LHRH receptors. In contrast to low molecular weight drugs, which are internalized inside the cells by diffusion, high molecular weight drugs are internalized by endocytosis (Minko et al., 2004). Endocytosis is a slow process and requires considerably higher drug concentration outside cells compared with diffusion. Therefore, toxicity of high molecular weight drugs is substantially lower compared with their low molecular weight precursors.

The rate of endocytosis is most likely the critical factor that limits cytotoxicity (in vitro) of high molecular weight CPT-PEG conjugates observed in the present study. In addition to targeting, moiety changes the mechanism of cellular uptake of prodrug to receptor-mediated endocytosis (Minko et al., 2004) and therefore enhances its toxicity. Normal cells that do not express LHRH receptors on their surface will utilize LHRH-containing delivery system by endocytosis, whereas cancer cells that overexpress the receptor will do so by receptor-mediated endocytosis. Consequently, toxicity of tumor-targeted DDS against cancer cells will be substantially
higher compared with normal cells, limiting adverse side effects of drug to normal cells. Based on the results of the present study, we expect that the difference in toxicities of 3×CPT-PEG-3×LHRH system between normal and cancer cells will be at least 70 times. In addition, high molecular weight of CPT-PEG-LHRH restricts its penetration through the blood-brain barrier, thus preventing possible adverse side effects on the brain and especially on the pituitary gland, which expresses LHRH receptor (Dharap et al., 2005).

It should be stressed that our data showed that PEG polymer itself provides passive targeting to solid tumor by the EPR effect, leading to the accumulation of high molecular weight substances in solid tumor (Minko et al., 2000a,b, 2004; Maeda, 2001; Fang et al., 2003; Greish et al., 2003; Minko, 2005). However, such passive targeting is attributed only to solid tumors and cannot provide targeting to spreading tumors and metastases. In contrast, LHRH targeting is equally applicable to solid tumor and a single cell. Therefore, proposed targeted prodrug has a potential against spreading tumors and metastases on the background of low toxicity to normal tissues. Such a wide range of targeting capabilities combined with high tumor toxicity and low toxicity to normal tissues is an essential characteristic of proposed novel multivalent delivery system that cannot be achieved by traditional anticancer approaches. Several targeting moieties can be added to a DDS using polymeric dendrimer approach (Kono et al., 1999; Patri et al., 2004; Choi et al., 2005). However, such a type of DDS may have an excessive molecular weight, which in turn can limit cellular internalization of the whole system and therefore decrease its toxicity. Our approach is based on the use of low molecular weight CA as a branched spacer that provides conjugation of multiple number of targeting moieties per one molecule of polymeric carrier with substantially lower molecular weight of the whole system compared with dendritic DDS.

The polymeric conjugates are inactive in their prodrug form during systemic delivery to the tumor and must release the drug from the delivery system to provide their anticancer activity. Therefore, the conjugates containing CPT molecules have been intentionally designed to possess an ester bond between the spacer and CPT. It is known that such a bond between PEG conjugate and CPT is hydrolyzed or broken by cellular enzymes such as esterase inside the cellular endosomes, leaving free drug for its action (Conover et al., 1997a; Greenwald, 2001; Greenwald et al., 2003b). On the other hand, the bond between the LHRH and PEG is an amide bond, which is more stable for the hydrolysis and in presence of enzymes. Our data showed that PEG polymer is internalized by cancer cells and distributed uniformly inside cells in the cytoplasm and nuclei. Although we did not analyze the pharmacokinetics of the studied compounds in vivo, based on our previous investigations of similar PEG polymer conjugates, one can expect substantial improvements in the pharmacokinetics with the increase in the molecular mass of the delivery system (Yu et al., 2005). Free low molecular weight of model compound was almost completely eliminated from the blood within the first 8 h after a single injection. At the same time, the level of the compound in the tumor was very low. The increase in the molecular weight of the compound by conjugation with PEG polymer substantially prolonged its circulation time over 24 h and increased blood drug concentration. Significant amounts of the compound were observed in the tumor up to 48 h after single injection. In addition, the conjugation of CPT to PEG substantially increases the stability of its active lactone form in the blood, preventing nonproductive premature biodegradation of the whole system (Conover et al., 1997b; Greenwald, 2001; Minko et al., 2002; Greenwald et al., 2004). Finally, all of these improvements substantially enhance antitumor activity of CPT after its conjugation with PEG polymer.

In the present investigation, we showed the possibility of combining in one targeted prodrug multiple copies of targeting moieties and anticancer drugs. For CPT, the maximal number of drug copies per one molecule of PEG conjugate was limited by the decrease in solubility of DDS when the number of CPT copies exceeded three. Addition of more copies of LHRH peptide seems not to improve the targeting. Similar targeted multifunctional approach can be applied to other drugs, targeting moieties, imaging agents, and other active components. It is also possible, using our approach, to combine several different drugs in one complex delivery system. Our novel approach is substantially different and involves the use of low molecular weight multifunctional spacer to obtain varying copies of the components. We are proposing the use of CA as a spacer, which consists of three carboxyl and one hydroxyl groups. Therefore, it offers the choice of conjugation with compounds having –OH, –COOH, or –NH2 functionality. In addition, each branch of multivalent spacer can be connected to similar multivalent branched spacer, providing the possibility of increasing the number of active ingredients without substantial increase in the molecular weight of the whole system. Present in vivo data confirmed the enhanced antitumor activity of the developed delivery systems containing multiple copies of targeting peptide and anticancer drug. Our approach can form a basis for a novel class of targeted tumor prodrugs—multivalent DDS with branched multiarmored spacers.

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Address correspondence to: Dr. Tamara Minko, Department of Pharma-


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