Novel polymeric prodrug with multivalent components for cancer therapy

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The list of non-standard abbreviations:

DDS – drug delivery system; LHRH – luteinizing hormone-releasing hormone; CPT – camptothecin; PEG – poly(ethylene glycol).

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 LHRH peptide, analog of luteinizing hormone-releasing hormone) and anticancer drug (camptothecin, CPT). 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 demonstrated 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, 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 hundred times more cytotoxic and substantially had enhanced antitumor activity when compared with the analogous non-targeted prodrug and prodrugs containing one or two copies of active components.

Introduction

Many anticancer drugs used in chemotherapy require modifications in order to: increase solubility, decrease adverse side effects, limit nonspecific activity, increase circulation time, modify biodistribution, etc. Various drug delivery systems have been developed to provide these modifications (Allen et al., 2000; Discher and Eisenberg, 2002; Ihre et al., 2002; Kataoka et al., 2001; Langer, 2001; Lasic and Papahadjopoulos, 1995; Torchilin, 2002; Uhrich et al., 1999; Zhang et al., 1996). Established drug delivery systems (DDS) primarily consist of polymeric conjugations or liposomal formulations (Allen and Cullis, 2004; Greenwald et al., 2003a; Minko et al., 2002; Rihova et al., 2001; 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 (Khandare et al., 2005; Kono et al., 1999; Malik et al., 2000). However, these novel polymers and their conjugates await proof of clinical safety and efficacy.

Targeted anticancer drug delivery systems offer further drug modifications and provide for the so-called "advanced targeted prodrug approaches" (Minko, 2004; Minko et al., 2004). In most cases such systems contain a nanocarrier or a water-soluble carrier conjugated with a targeting moiety and an anticancer drug (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. 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 detectable in normal

visceral organs (Dharap and Minko, 2003; Dharap et al., 2003; Dharap et al., 2005; Furui et al., 2002; 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.

Poly(ethylene glycol) (PEG) polymer is widely used as a carrier for drug delivery systems (Greenwald, 2001). PEG is a water-soluble nonionic polymer approved by FDA for pharmaceutical applications. Due to 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 (Caliceti et al., 2001). Successful bioconjugation depends upon the chemical nature, structure, molecular weight, steric hindrance and reactivity of the biomolecule as well as of the polymer. In most prodrug conjugations a variety of spacers can be incorporated between polymer and biomolecules; as they can offer a chemical flexibility and can be hydrolyzed to release the bioactive component (Kopecek et al., 2000; Kopecek et al., 2001). The suitability of a spacer molecule depends upon 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 paper 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 luteinizing hormone–releasing hormone) 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 might be utilized for the delivery of different drugs and imaging agents, as well as other than 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 whole system, steric hindrance of components, etc. 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.

Material and Methods

Cell line. The human ovarian carcinoma A2780 cell line was obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center, PA). Cells were cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FCS) (HyClone, Logan, UT). Cells were grown at 37º C in a humidified atmosphere of 5% CO_2 (v/v) in air. All 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 (DIEA) and 4-(methylamino)pyridine (DMAP) were obtained from Sigma Chemical Co. (Atlanta, GA), bis (2-carboxyethyl) poly(ethylene glycol) M_w~3,000 Da polymer and fluorescein isothiocyanate (FITC) were obtained from Fluka (Allentown, PA). CPT (sodium salt CPT: CAS number: 25387-67-1) 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 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 (Dharap et al., 2005; Minko et al., 2004; Minko et al., 2000a; Minko et al., 2000b; Minko et al., 2002) we selected PEG polymer with molecular weight 3000 which shows only moderate decrease in bioavailability and cytotoxicity of CPT while it improves drug pharmacokinetics. Such relatively low molecular weight polymer does not provide for effective passive tumor targeting by the 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-citric acid conjugates (compound 3 in Fig. 2b). $α,ω$ bis PEG$_{3000}$-citric acid conjugate was synthesized using one step procedure (Fig. 2). Citric acid (2 in Fig. 2b, CAS number: 77-92-9, 2-hydroxy-1,2,3-propanetricarboxylic acid)

contains one hydroxyl and three carboxyl functional reactive groups for chemical conjugation. Briefly, bis (2-carboxyethyl) PEG (1 in Fig. 2b) (100 mg, 0.033 mM) and 2 moles of citric acid (12.8 mg, 0.066 mM) were dissolved in 5 ml of anhydrous dimethylformamide (DMF) and 20 ml of anhydrous dichloromethane (DCM). To this solution *N*-(3- dimethylaminopropyl)-*N*-ethylcarbodiimide HCl (EDC.HCl, 13 mg, 0.0678 mM) was added 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 carbodiimide urea formed during the reaction was removed by filtration. The unreacted citric acid and EDC.HCl were removed by dialysis using Spectra/Por membrane (M_W cut off ~ 2,000 Da) in DMF as a solvent. Further purification of the α,ω bis PEG-citric acid conjugate was carried out using size exclusion Sephadex G 10 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) PEG$_{3000}$-citric acid conjugate (3 in Fig. 2b) (M_W~3,382.24 Da, 50 mg, 0.0147 mM) and CPT (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. To the above solution EDC.HCl (3 mg, 0.0156 mM) was added 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 carbodiimide urea formed during the reaction was removed by filtration. The unreacted CPT and EDC.HCl were removed by dialysis using Spectra/Por dialysis membrane (M_W cut off~2,000 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. ^{1}HNMR spectra of α,ω bis (2-carboxyethyl) PEG-CPT conjugates were recorded on Varian 400 MHz spectrophotometer using DMSOd6 as a solvent. CPT protons correspond to (1) δ 0.9 CH$_3$ (t), δ 1.9 CH$_2$ (m), δ 5.35 CH$_2$ (s), δ 5.5 CH$_2$ (s), 6.7 CH (s), δ 7.4 –CH (s), δ 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 corresponds 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) PEG$_{3000}$-citric acid-CA-1CPT conjugate (M$_w$~3,729 Da, 50 mg, 0.0134 mM) and peptide LHRH-NH$_2$ (18 mg, 0.013 mM) were dissolved in 3 ml of anhydrous DMSO and 12 ml of anhydrous DCM. The reaction mixture was allowed to stir for 30 min. To the above solution EDC. HCl (3.0 mg, 0.0134 mM) was added as a condensing agent and DMAP (1.0 mg, 0.008 mM) was used a catalyst. The reaction was stirred continuously for 48 h at room temperature. The carbodiimide urea formed during the reaction was removed by filtration. The unreacted CPT and EDC.HCl were removed by dialysis using Spectra/Por dialysis membrane (M$_w$ cut off~2,000 Da) using DMSO as solvent. The conjugate was dried under vacuum at room temperature. In addition, polymer conjugates with two and three copies of peptide LHRH-NH$_2$ were prepared by using two and three molar ratios of LHRH-NH$_2$ and EDC.HCl respectively. Molecular weights of conjugates were recorded on MALDI/TOF (Fig.3b) and presence of LHRH peptide was analyzed by UV spectroscopy (Fig.3c).

α,ω bis (2-carboxyethyl) PEG-FITC conjugate. Hydroxyl groups of FITC were condensed with the carboxyl group of bis (2-carboxyethyl) PEG using EDC.HCl as coupling agent. Briefly, bis PEG (50 mg, 0.016 mM) and FITC (6.2 mg, 0.016 mM) were

dissolved in anhydrous DMSO (2 ml) and anhydrous DCM (10 ml). 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 carbodiimide salt. The conjugate was washed with excess acetone three times to remove free FITC and the conjugate was precipitated in diethyl ether. Further, bis PEG-FITC conjugate was purified by dialysis using Spectra/Por dialysis membrane (M_W cut off ~2,000 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; Dharap et al., 2005) by American Peptide Company, Inc. (Sunnyvale, CA). Rhodamine red succinimidyl ester was covalently conjugated with LHRH peptide having NH_2 at the terminal. Briefly, LHRH (5.0mg, 0.0037 mM) and LHRH-NH_2 (3 mg, 0.0039 mM) was dissolved in anhydrous DMF (2.0 ml) and added DIEA (4.0 ml) to adjust alkaline pH and maintain amine group in non protonated 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 non conjugated LHRH and rhodamine red ester.

UV analysis of LHRH and LHRH containing conjugates. 1 mg of standard LHRH-NH_2, 3xCPT-PEG (no LHRH), 3xCPT-PEG-1xLHRH, 3xCPT-PEG-2xLHRH, 3xCPT-PEG-3xLHRH conjugates were dissolved in 1ml of deionized water and UV spectra were recorded from 250 nm 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 40 and number of flashes 03. 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 mg/ml. Amount of CPT in bis (2-carboxyethyl) PEG-citric acid-CPT conjugates with one, two and three copies of CPT were 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 citric acid spacer. Bis PEG polymer (M_W~3,000 Da) linked to CA and/or CPT molecule structures were built using ChemDraw 9.0 Pro (Cambrigesoft 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. 4a, b). The bioconjugate with PEG was built to represent seven ethylene repeat ($-CH_2-O-CH_2-$) units. The settings for energy minima and molecular dynamics were: order 1.000 Step Interval: 2.0 fs, Frame Interval: 10 fs, Terminate After 10000 Steps, Heating/Cooling Rate: 1.000 Kcal/atom/ps, Target Temperature: 300º K.

In vitro **cellular entry of conjugates.** A2780 human ovarian cancer cells were seeded in a 6-wells culture plate ($1x10^4$ cells per 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 ^{O}C with CPT, 3xCPT-PEG and 3xCPT-PEG-3xLHRH conjugate. Conjugates were dissolved in PBS buffer (pH 7.4), whereas free CPT was first dissolved

in 5 % of DMF, and then added to PBS buffer. 100 µL of supernatant was removed at interval of 0, 5, 10, 15, 30, 45, 60 min, 3, 6, and 24 h. Equal volume of media was replaced to each well. The supernatant was centrifuged at ~12,000 x g for 5 minutes using Eppendorf centrifuge 5415D to remove the cellular debris. Cellular uptake for conjugate 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 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (Dharap and Minko, 2003; Fang et al., 2003; Greish et al., 2003; Maeda, 2001; Minko et al., 2003).

In vivo **antitumor activity.** Previously developed mouse model of human ovarian carcinoma xenografts was used (Dharap et al., 2005; Minko et al., 2000a; Minko et al., 2000b). Briefly, 2 x 10^6 A2780 human ovarian carcinoma cells were subcutaneously transplanted into the flanks of female athymic nu/nu mice. When the tumors reached a size of about 1 cm^3 (15-20 days after transplantation), mice were separately treated intraperitoneally with saline (control), CPT, and 1xCPT-PEG, 2xCPT-PEG, 3xCPT-PEG, 1xCPT-PEG-1xLHRH, 2xCPT-PEG-1xLHRH, 3xCPT-PEG-1xLHRH, 3xCPT-PEG-2xLHRH, 3xCPT-PEG-3xLHRH conjugates. The dose of all substances (10 mg/kg for the single injection) corresponded to the maximum tolerated dose of CPT. Equivalent CPT concentration was 3, 3.8 and 5.6 mg/kg for conjugates containing 1, 2 and 3 copies of CPT respectively. Maximum tolerated dose was estimated in separate experiments based on animal weight changes after the injection of increasing doses of the drugs as previously described (Dharap et al., 2005; Minko et al., 2000a; Minko et al., 2000b).

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 *in vitro* and *in vivo* experiments were performed in quadruplicate. The results are expressed as mean ± s.d. from 4-8 independent measurements. Statistical analysis was performed as a one-way analysis of variance (ANOVA) 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; Dharap et al., 2005; Minko et al., 2004) and others (Furui et al., 2002) have reported that receptors for luteinizing hormone-release hormone (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 when compared to 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 which 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; Dharap et al., 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 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 1, 2, and 3 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 1, 2, and 3 CPT copies respectively. In contrast, free camptothecin is practically insoluble in aqueous solutions.

 The details of the synthesis are summarized below.

   α,ω bis (2-carboxyethyl) PEG-citric acid conjugate. The PEG polymer reported here was *α,ω bis* (2-carboxyethyl) PEG ($M_W \sim 3,000$ Da) and was a di-acid having molecular formula $HOOCCH_2CH_2CONH(CH_2CH_2O)_nCH_2CH_2NHCOCH_2CH_2COOH$. Two moles of citric acid were coupled (i.e. hydroxyl group of CA) with one mole of carboxyl terminated bis PEG using *N*-(3- dimethylaminopropyl)-*N*-ethylcarbodiimide HCl as coupling agent to form tri-carboxylate groups at both terminals (Fig. 2b). Multi functional citric acid was used as a spacer to decrease the steric hindrance and increase the reactivity of PEG-CA conjugate with CPT.   The resulting bis PEG-citric acid conjugate was

purified using molecular weight cut off dialysis membrane (M_W~2,000 Da) and size exclusion G10 sephadex column. bis PEG-citric acid conjugate contained total number of six carboxylate groups for further conjugation with multiple copies of CPT-OH and LHRH-NH$_2$.

***α,ω bis (2-carboxyethyl) PEG-citric acid-CPT conjugate*.** Hydroxyl group at the position 20 of CPT was covalently coupled with carboxyl groups of bis PEG-citric acid to form a degradable ester bond. One, two and three copies of CPT per bis PEG-citric acid conjugate was obtained by reacting one, two and three moles of CPT with one mole of polymer respectively (Fig. 2c). Similarly, molar ratio of *N*-(3- dimethylaminopropyl)-*N*-ethylcarbodiimide HCl and dimethyl amino pyridine (DMAP) were varied. Polymer conjugate with one, two and three copies of CPT demonstrated substantially higher aqueous solubility compared to free CPT, which is not soluble in aqueous solutions. The formation of CPT-bis PEG conjugates were confirmed by proton (^{1}HNMR) and ^{13}C (^{13}CNMR) nuclear magnetic resonance. The aromatic peaks in Fig. 3a indicate the presence of CPT molecules along with broad peaks for bis PEG, while ^{13}C NMR confirmed the formation of ester bond between CPT and PEG-citric acid (data not shown). In addition, ^{13}CNMR 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 due to the presence of multiple carboxyl groups for conjugation of drug with polymer. The integration ratios for PEG peaks were decreased with increased number of copies of

CPT. MALDI/TOF (PEbiosystems Vovager Systems 6080) showed increased molecular weight for CPT-PEG conjugate. Further, the concentration of CPT in the 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 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 ~360 nm and excitation ~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 wavelengths to that of CPT up to concentrations of 1.0 mg/ml. The detection limit of CPT by fluorescence spectroscopy was 0.0007 mM of concentration.

α,ω bis (2-carboxyethyl) PEG-citric acid-CPT-LHRH conjugate. LHRH analog, Lys6–des-Gly10–Pro9-ethylamide (Gln–His–Trp–Ser–Tyr–DLys–Leu–Arg–Pro–NH–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-citric acid-CPT-conjugate to form a non degradable amide bond (Fig. 2d). The amount of LHRH to obtain one, two and three copies per bis PEG-citric acid-CPT conjugate was manipulated on the molar basis. Similarly, the molar ratio of EDC.HCl and DMAP were varied. The concentration of CPT in the each conjugate was estimated by fluorescence spectroscopy at excitation 360 nm and emission 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. Due to high molecular weight constraints, the three dimensional confirmation of polymers along with conjugated biomolecules is not being elucidated. It was thought worthwhile to build a molecular confirmation and evaluate energy minima's, stable confirmations and molecular dynamics for synthesized conjugates (Fig.4). The minima represented the energies (kcal) indicating degree of conformational freedom for 7 repeating units of bis PEG polymeric chain along with citric acid as a spacer. In addition, molecular dynamics confirmations were established for conjugates with one, and three copies of CPT molecules. Figure 4a shows the molecular dynamic confirmation for the bis PEG-CA conjugate containing three copies of CPT, whereas Fig. 4b represents distance and adjunct molecular alignments for the same conjugate. Distance between the first and last carbon atom for molecular dynamic structure of free CPT was 8.887 Å 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-1xCPT conjugate without citric acid 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 demonstrate the presence of targeting LHRH-NH$_2$ peptide in the conjugate, UV spectra were recorded for LHRH-NH$_2$ peptide alone, 3xCPT-PEG (no LHRH), 3xCPT-PEG-1xLHRH, 3xCPT-

PEG-2xLHRH, 3xCPT-PEG-3xLHRH conjugates (Fig.3c). The increase in UV absorbance at ~280 nm for conjugates with 1, 2 or 3 copies of LHRH peptide indicates higher amount of peptide incorporation in the conjugate.

In vitro cellular uptake of 3xCPT-PEG and 3xCPT-PEG-3xLHRH conjugates. To show internalization of conjugates by ovarian cancer cells, we examined the cellular uptake of CPT, 3xCPT-PEG and 3xCPT-PEG-3xLHRH 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 minutes and reached a plateau. In contrast, free CPT was internalized by the cells significantly slower when compared with 3xCPT-PEG conjugate. Only about 12% of the applied dose of CPT entered the cells within 30 min. Moreover, it should be noted that due to very low aqueous solubility of CPT, the drug was first dissolved in 5 % of DMF and further added to PBS buffer having pH 7.4. Addition of three copies of targeting moiety (synthetic analog of LHRH peptide) to the above polymer substantially enhances internalization of the conjugate. Where as final intracellular concentration of 3xCPT-PEG-3xLHRH conjugate was almost three times higher when 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, while 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.

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In vitro cytotoxicity. Conjugation of CPT with PEG polymer resulted in high molecular weight complex and a decrease in its toxicity (increased IC_{50} 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 whole system in 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 in 5.3, 8.87 and 12.9 times respectively when compared with 1xCPT-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 3xCPT-PEG-2xLHRH and 3xCPT-PEG-3xLHRH conjugates were 30 and 77 times more toxic against human ovarian carcinoma cells when compared with non-targeted 1xCPT-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. While 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 3 copies of CPT and 2-3 copies of LHRH), even non targeted polymeric CPT-PEG conjugates with 1-3 copies of CPT were substantially more effective in terms of suppressing tumor growth (please compare curves 2-4 with curve 1 on Fig. 6 and 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 Enhanced Permeability and Retention (EPR) effect (Minko, 2005; Minko et al., 2004; Minko et al., 2000a; Minko et al., 2000b). Further enhancement of antitumor activity was achieved by "active targeting" when one to three copies of tumor targeting moiety were added to the multicomponent delivery system (please compare curves 6-9 with curves 2-4).

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 (Dharap and Minko, 2003; Dharap et al., 2003; Dharap et al., 2005; Furui et al., 2002; 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 when compared to 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 which 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 when compared with diffusion. Therefore, toxicity of high molecular weight drugs is substantially lower when compared to their low molecular weight precursors. The rate of endocytosis is most likely the critical factor which limits cytotoxicity (in vitro) of high molecular weight CPT-PEG conjugates observed in the present study. Including 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 which do not express LHRH receptors on their surface will utilize LHRH-containing delivery system by endocytosis, while cancer cells that overexpress the receptor – by receptor-mediated endocytosis. Consequently, toxicity of tumor-targeted

DDS against cancer cells will be substantially higher when 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 3xCPT-PEG-3xLHRH 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 brain and especially on pituitary gland which express 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 (Fang et al., 2003; Greish et al., 2003; Maeda, 2001; Minko, 2005; Minko et al., 2004; Minko et al., 2000a; Minko et al., 2000b). 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 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 drug delivery system using polymeric dendrimer approach (Choi et al., 2005; Kono et al., 1999; Patri et al., 2004). However, such type of DDS might 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 citric acid as a branched spacer which provides

conjugation of multiple number of targeting moieties per one molecule of polymeric carrier with substantially lower molecular weight of the whole system when 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 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 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 non-productive premature biodegradation of whole system (Conover et al., 1997b; Greenwald, 2001; Greenwald et al., 2004; Minko et al., 2002). Finally all 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 maximum 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 exceeds 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 citric acid 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 –NH$_2$ 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 multi-armed spacers.

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Footnotes

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Legends for figures

Figure 1. Localization of LHRH and PEG in cancer cells expressed LHRH receptors. Human ovarian carcinoma A2780 cells were incubated for 24 h with PEG polymer and LHRH peptide labeled with fluorescein isothiocyanate (FITC, green fluorescence) and rhodamine (red fluorescence) respectively. In addition, cell nuclei were labeled with cell nuclear-specific dye (Hoechst 33258) with blue fluorescence. Fluorescence microscopy analysis showed that LHRH peptide localized in the plasma membrane and outer part of cellular cytoplasm, while PEG polymer of this size equally penetrated cellular cytoplasm and nuclei. Superposition of images allows for detecting of co-localization of LHRH with PEG (yellow color) and nuclear localization of PEG (cyan).

Figure 2. Synthesis of targeted multivalent anticancer prodrug. The system **(a)** contains bis poly(ethylene glycol) (PEG) polymer as a carrier, one, two or three copies of LHRH peptide as a targeting moiety and one, two or three copies of camptothecin (CPT) as an anticancer drug. **(b)** α,ω bis PEG$_{3000}$-citric acid conjugate (3) was synthesized using one step procedure by conjugation of 1 M of bis (2-carboxyethyl) PEG (1) with 2 M of citric acid [CAS number: 77-92-9, 2-hydroxy-1,2,3-propanetricarboxylic acid] (2). Bis PEG citric acid conjugate (3) was coupled with one, two and three moles of CPT (4) to obtain α,ω-bis (2-carboxyethyl) PEG-citric acid-CPT conjugates (**c**, 5, 6, 7). Further, one, two and three moles of LHRH (8) was conjugated with c: 5, 6, and 7 to obtain α,ω-bis (2-carboxyethyl) PEG-citric acid-CPT-LHRH conjugates (**d**, 5a, 6a, 7a) having one, two and three copies of CPT (4) and LHRH (8).

Figure 3. Characterization of synthesized polymer conjugates: **(a)** ^{1}HNMR spectra for α,ω-bis (2-carboxyethyl) PEG-citric acid-CPT conjugate in DMSO d6 400 MHz. Proton from δ 3.2 to 3.8 broad peaks represents for bis (2-carboxyethyl) PEG and peaks from δ 5.35 to 8.2 corresponds to protons of CPT. **(b)** MALDI/TOF for α,ω-bis (2-carboxyethyl) PEG-citric acid-2xCPT-2xLHRH conjugate. Molecular weight for the conjugate was observed to be about 5,363 Da. For bis PEG-CA with one copy of CPT molecular weight was estimated as 3,413 Da (Spectra not shown). **(c)** UV spectra for LHRH and LHRH conjugates with bis PEG-CPT. Free LHRH (standard) and conjugates containing 1, 2 and 3 copies of LHRH were dissolved in deionized water and UV spectra were recorded. The increase in UV absorbance at ~280 nm for conjugates with 1, 2 or 3 copies of LHRH peptide indicates higher amount of peptide incorporated in the conjugate. **(d)** cellular entry of 3xCPT-PEG and 3xCPT-PEG-3xLHRH conjugates in A2780 ovarian carcinoma cells. Addition of LHRH peptide substantially enhanced cellular internalization of conjugate within 30 minutes compared to 3x CPT PEG conjugate.

Figure 4. Molecular modeling studies for seven repeating units of bis PEG polymer with two copies of citric acid as a spacer attached with three copies of CPT. Polymeric structures were built in Chemdraw software and allowed to undergo conformational stabilization for **(a)** energy minima and **(b)** molecular dynamics. The stabilized structures were exported to RasTop software to estimate the distances between first and last carbon atoms (here C1 and C94). The distance was measured to be 12.23 Å for energy minimized bis PEG-CA-3xCPT molecule, while it was 13.74 Å for molecularly dynamic structure. The above studies represent theoretical understanding of molecular

confirmations and structural stability of high molecular weight polymers, covalently conjugated to multiple copies of drug molecules.

*$P < 0.05$ when compared with free CPT

[†]$P < 0.05$ when compared with 3xCPT-PEG

Figure 5. Inclusion of several copies of anticancer drug (camptothecin, CPT) and targeting moiety (synthetic analog of LHRH peptide) substantially enhances toxicity of prodrug towards human ovarian cancer cells. Human ovarian carcinoma A2780 cells were separately incubated with 45 different concentrations of each drug. IC_{50} dose (dose that kills 50% of cancer cells) was measured for each drug. Means ± s.d. are shown.

[*]$P < 0.05$ when compared with free CPT

[†]$P < 0.05$ when compared with 1xCPT-PEG

Figure 6. Inclusion of several copies of anticancer drug (camptothecin, CPT) and targeting moiety (synthetic analog of LHRH peptide) substantially enhances antitumor activity of prodrug towards xenografts of human ovarian tumor. A2780 human ovarian carcinoma cells were subcutaneously transplanted into the flanks of female athymic nu/nu mice. When the tumors reached a size of about 1 cm^3 (15-20 days after transplantation), mice were treated once with each drug conjugates. The dose of all substances (10 mg/kg for the single injection) corresponds to the maximum tolerated dose of CPT. Equivalent CPT concentration was 3, 3.8 and 5.6 mg/kg for conjugates containing 1, 2 and 3 copies of CPT respectively. Tumor size was measured at 6, 12, 18, 24, 48, 72, and 96 h after the treatment. Means ± s.d. are shown.

*$P < 0.05$ when compared with control

†$P < 0.05$ when compared with free CPT
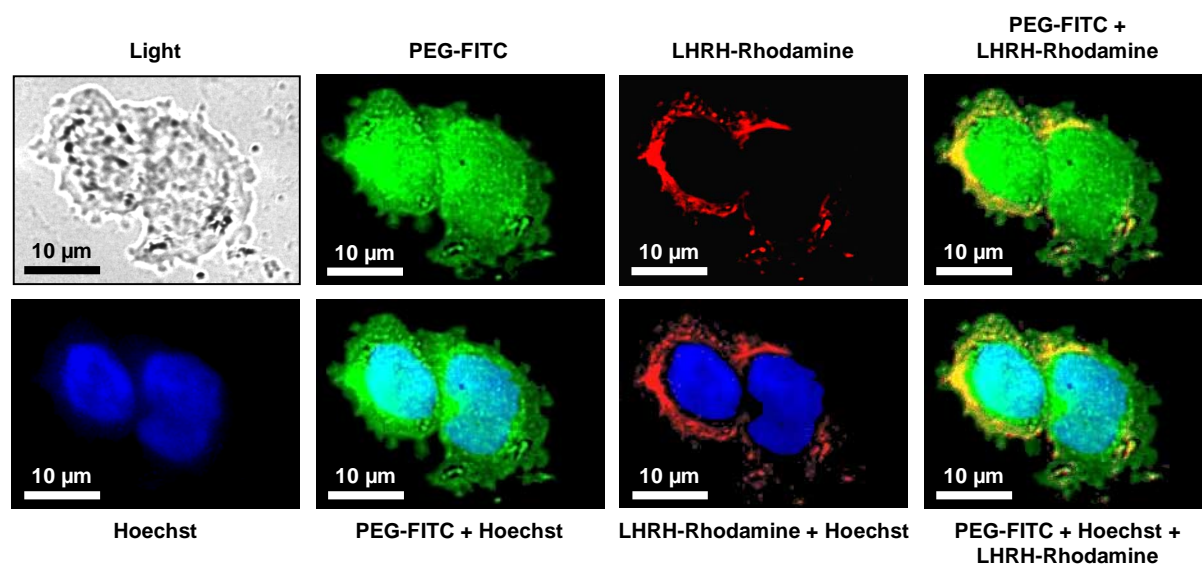
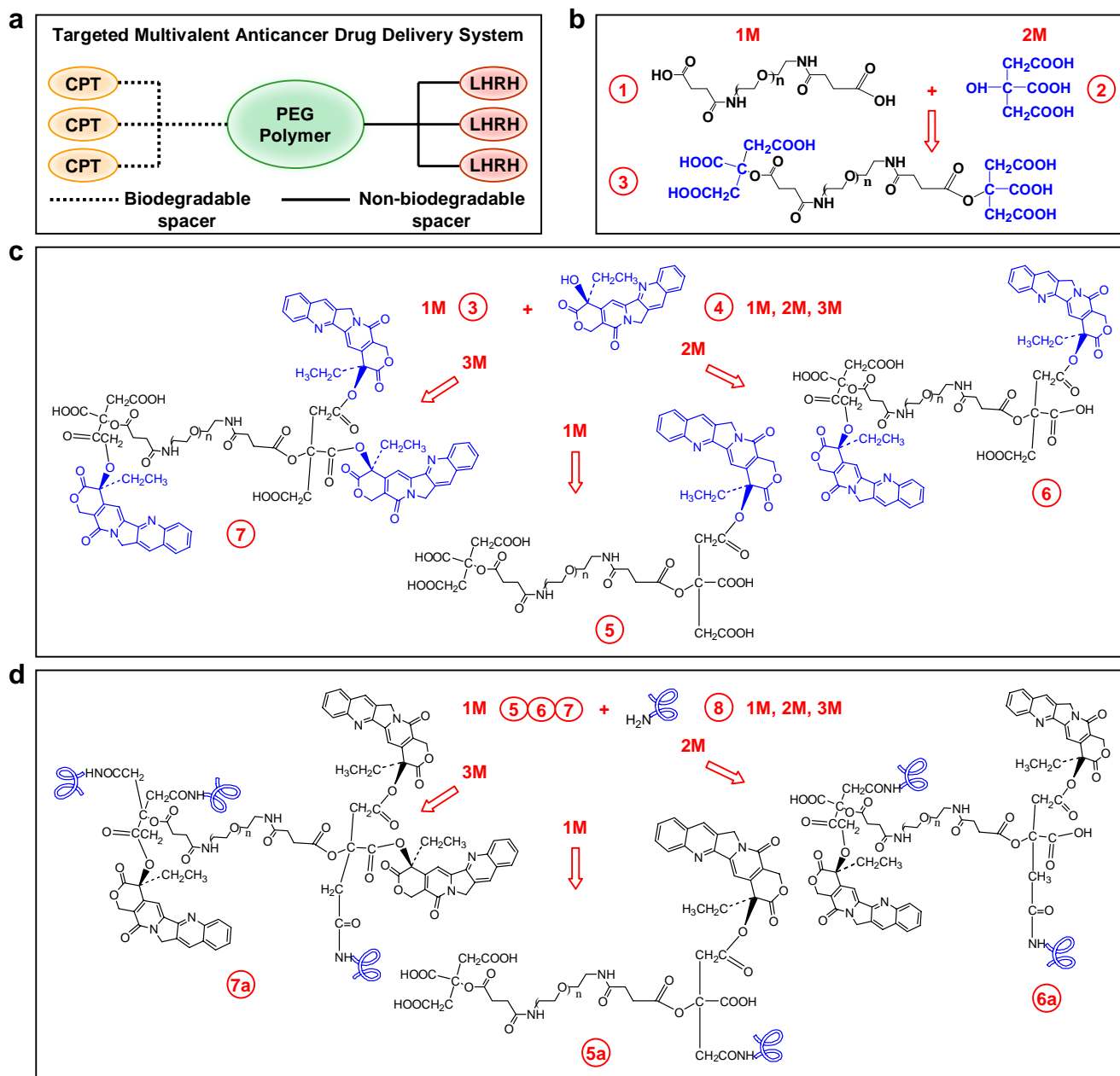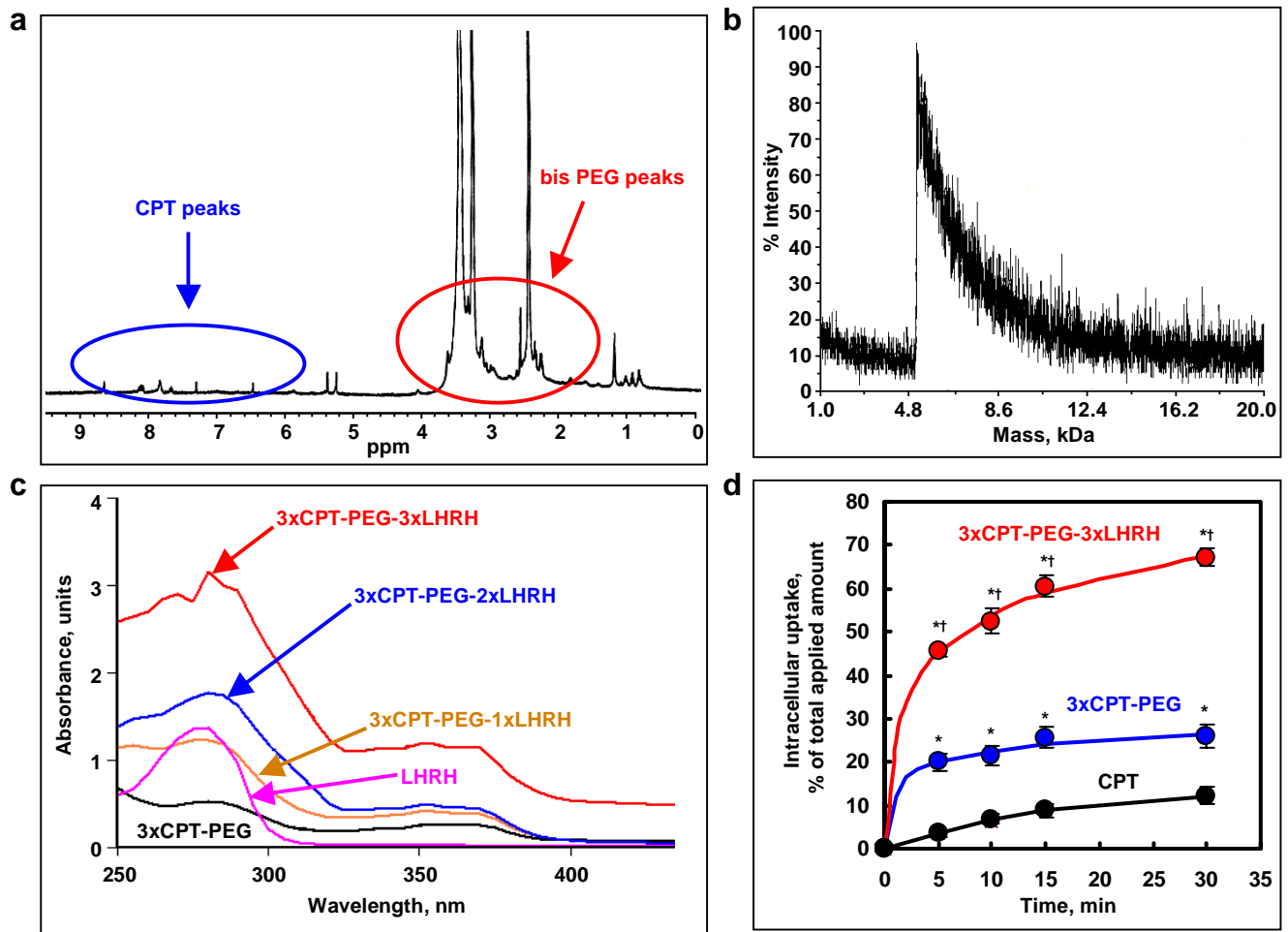‡$P < 0.05$ when compared with 1xCPT-PEG

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6