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Prodrugs Forming High Drug Loading Multifunctional Nanocapsules for Intracellular Cancer Drug Delivery

Youqing Shen,*,†,‡ Erlei Jin,‡ Bo Zhang,‡ Caitlin J. Murphy,§ Meihua Sui,† Jian Zhao,† Jinqiang Wang,† Jianbin Tang,† Maohong Fan,‡ Edward Van Kirk,§ and William J. Murdoch§

Center for Bionanoengineering and the State Key Laboratory for Chemical Engineering, Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou, China 310027, Department of Chemical and Petroleum Engineering and Department of Animal Science, University of Wyoming, Laramie, Wyoming 82071

Received November 7, 2009; E-mail: shenyq@zju.edu.cn

Abstract: Anticancer drugs embedded in or conjugated with inert nanocarriers, referred to as nanomedicines, show many therapeutic advantages over free drugs, but the inert carrier materials are the major component (generally more than 90%) in nanomedicines, causing low drug loading contents and thus excessive uses of parenteral excipients. Herein, we demonstrate a new concept directly using drug molecules to fabricate nanocarriers in order to minimize use of inert materials, substantially increase the drug loading content, and suppress premature burst release. Taking advantage of the strong hydrophobicity of the anticancer drug camptothecin (CPT), one or two CPT molecule(s) were conjugated to a very short oligomer chain of ethylene glycol (OEG), forming amphiphilic phospholipid-mimicking prodrugs, OEG-CPT or OEG-DiCPT. The prodrugs formed stable liposome-like nanocapsules with a CPT loading content as high as 40 or 58 wt % with no burst release in aqueous solution. OEG-DiCPT released CPT once inside cells, which showed high in vitro and in vivo antitumor activity. Meanwhile, the resulting nanocapsules can be loaded with a water-soluble drug—doxorubicin salt (DOX·HCl)—with a high loading efficiency. The DOX·HCl-loaded nanocapsules simultaneously delivered two anticancer drugs, leading to a synergetic cytotoxicity to cancer cells. The concept directly using drugs as part of a carrier is applicable to fabricating other highly efficient nanocarriers with a substantially reduced use of inert carrier materials and increased drug loading content without premature burst release.

Introduction

Most anticancer drugs have poor water solubility, rapid blood clearance, low tumor selectivity, and severe side effects for healthy tissues. Nanosized vehicles[1] including liposomes,[2] water-soluble polymers,[3] dendrimers,[4] vesicles,[5] polymer nanoparticles,[6,7] and some inorganic materials[8] are thus used as the carriers of the drugs to greatly enhance their water solubility and stability,[9] prolong their circulation in blood compartments,[10] target cancerous tissues by passive accumulation[11] via tumors' enhanced permeability and retention (EPR) effect[12] and using such targeting groups as folic acid[13,14] and EGF.[15] Therefore, by increasing drug bioavailability at sites of action, drugs in the nanocarriers, referred to as nanomedicines,[1] have shown thera-

† Zhejiang University.

‡ Department of Chemical and Petroleum Engineering, University of Wyoming.

§ Department of Animal Science, University of Wyoming.

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peutic advantages including better efficacy against resistant tumors and fewer side effects over free drugs.[14,16]

These drug carriers are typically inert, and their sole role is to make the vehicles. For instance, the lipid and cholesterol in conventional liposomes have no other functions but to make the vesicles carrying drugs. The amphiphilic block copolymers used to make nanoparticles are simply for forming the core−shell structure encapsulating hydrophobic drugs in their core.[17] These inert carriers, however, are the major component while the therapeutic drugs are the minor component in most nanomedicines. In nanoparticles or liposomes, the drug contents are generally not greater than 10%[7,18] to minimize the initial drug release in the blood compartments (premature burst release). In polymer−drug conjugates, the drugs generally represent only a few percent to keep the conjugates water-soluble.[11,19] For instance, the CPT content in its conjugate to a PEG with a molecular weight of 40 kDa was only 0.86 wt % (conjugated with one CPT) or 1.72% (with two CPT).[11,20]

Accordingly, the inherent drawback of these nanomedicines is their low drug loading capacity. A large amount of a carrier has to be used to administer a needed dose of a drug. For parenteral applications, repeated administrations of high doses of these excipients may cause systemic toxicity such as lipotoxicity and impose an extra burden for the patients to excrete the carriers.

In an amphiphilic block copolymer or lipid, the hydrophilic and lipophilic parts are connected to have balanced amphiphilicity for forming micelles or liposomes. Most anticancer drugs are very hydrophobic. We thus proposed that using a hydrophobic drug as the hydrophobic part of the block copolymer or lipid would minimize the use of other inert materials and thus increase the drug loading content. Herein, we demonstrate this concept using a hydrophobic drug CPT to replace the fatty acid(s) in phospholipids, forming a phospholipid-mimic structure (Scheme 1, OEG-DiCPT). CPT is extremely hydrophobic with a water solubility of about 3 μg/mL. A very short nonionic OEG chain with only eight repeating units was selected as the water-soluble part to maximize the drug loading content and also to lower the critical vesicle formation concentration (CV_fC). A β-thioester bond was used as the linker because it is easily hydrolyzed by esterase, which is abundant in cells. For comparison, the OEG conjugated with one CPT molecule (OEG-CPT) was also synthesized. The amphiphilic lipid-like CPT prodrugs formed liposome-like nanocapsules in aqueous solution. The nanocapsules had two roles: As a nanocarrier of CPT, it achieved high CPT loading contents without premature drug release. Additionally, the nanocapsules can serve as carriers of other anticancer drugs, such as doxorubicin (DOX·HCl), for combination therapy.

Results and Discussion

Synthesis of the Prodrugs and Formation of Prodrug Nanocapsules. The syntheses of the CPT prodrugs are shown in Scheme 2. In the synthesis of OEG-DiCPT, the short OEG chain with two terminal carboxylic acids was first synthesized via two Michael addition reactions. The reaction of the carboxylic acids with CPT was first tried using DCC/DMAP-catalyzed esterification, but the reaction was incomplete, probably due to steric hindrance. The carboxylic acids were then converted to acyl halides, which reacted with two CPT

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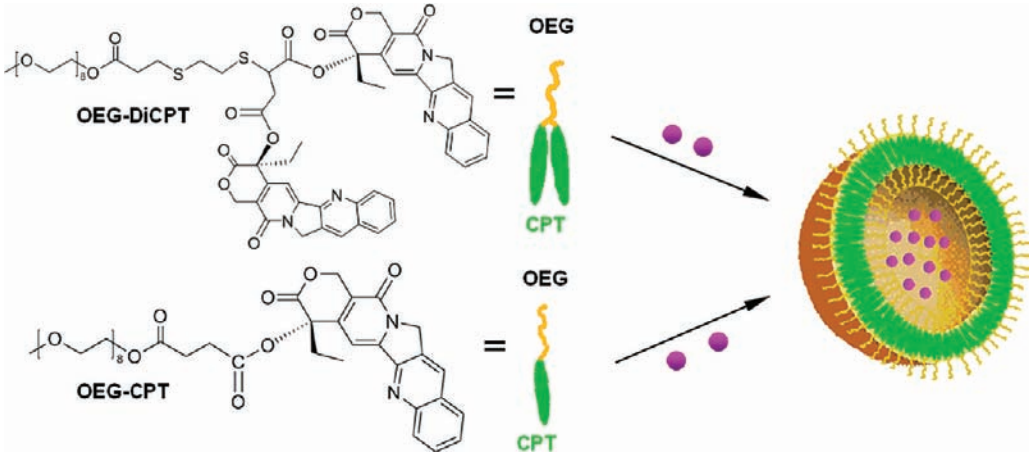
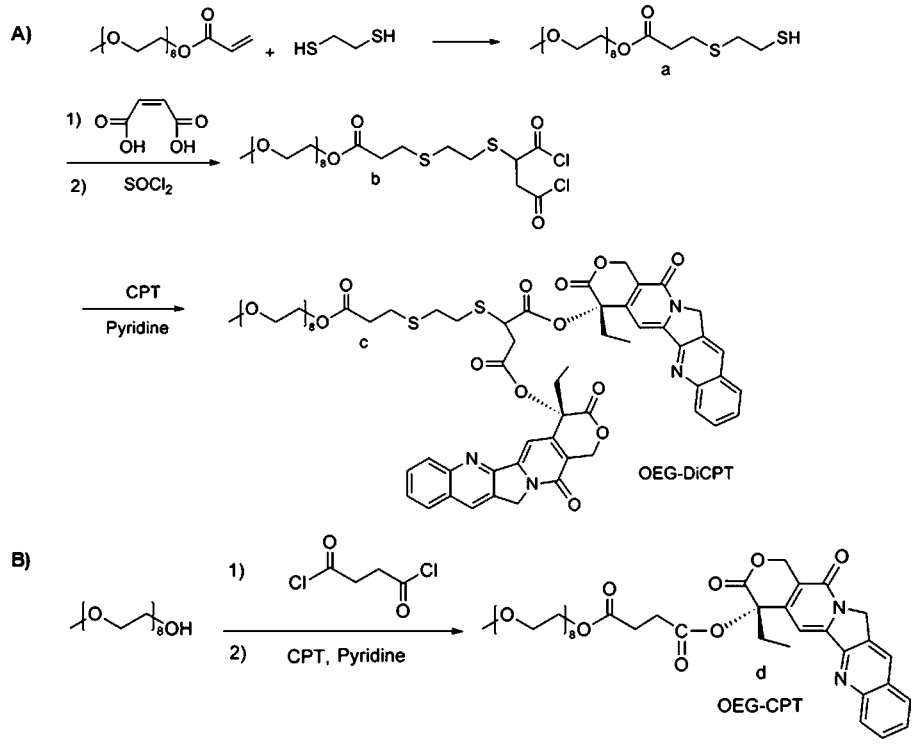
Scheme 1. Amphiphilic Camptothecin (CPT) Prodrugs (OEG-CPT and OEG-DiCPT) and Their Self-Assembly into Nanocapsules To Load Other Drugs (●) (Hydrophobic Membrane is Solely Made of CPT Moieties)



Scheme 2. Synthesis of OEG-DiCPT and OEG-CPT



molecules completely to produce OEG-DiCPT with few impurities. The structure of the resulting OEG-DiCPT was confirmed by NMR and HPLC. It had a water solubility of 1.64 mg/mL, or 0.96 mg/mL in terms of CPT, which is 300 times that of pristine CPT. The CPT's lactone ring structure was retained in the OEG-DiCPT as confirmed by NMR and was stable in aqueous solution as evidenced by a very similar cytotoxicity even after remaining in aqueous solution for long periods (Supporting Information, Figure S6). For comparison, OEG conjugated with one CPT molecule was also synthesized by direct ester formation of CPT with the carboxylic acid of the OEG (Scheme 2B).

OEG-DiCPT and OEG-CPT formed nanosized particles with critical vesicle formation concentrations of 45 and 21 μg/mL, respectively (Supporting Information Figure S1). Interestingly, OEG-CPT had a lower CV_fC than OEG-DiCPT even though it had a lower fraction of the hydrophobic part. This is probably because the planar CPT molecules could pack tightly in OEG-CPT but could not in OEG-DiCPT due to steric hindrance. The structure of the particles was observed by TEM (Figure 1). The OEG-DiCPT particles were loaded with cisplatin or phosphotungstic acid (PTA) as dyes. Cisplatin is not very water-soluble and thus stains the hydrophobic region. PTA is water-soluble and thus stains the hydrophilic region. The OEG-DiCPT particles dyed with cisplatin had a dark membrane surrounding a less colored core (Figure 1a), while those stained with PTA had a dark core (Supporting Information Figure S2), suggesting a vesicular structure. Similarly, OEG-CPT particles stained with PTA had a very dark core (Figure 1d). These results indicate that the amphiphilic prodrugs OEG-DiCPT and OEG-CPT indeed formed about 100−200 nm liposome-like nanocapsules. The size of the OEG-DiCPT nanocapsules in aqueous solution, measured by dynamic laser light scattering (DLS), was about

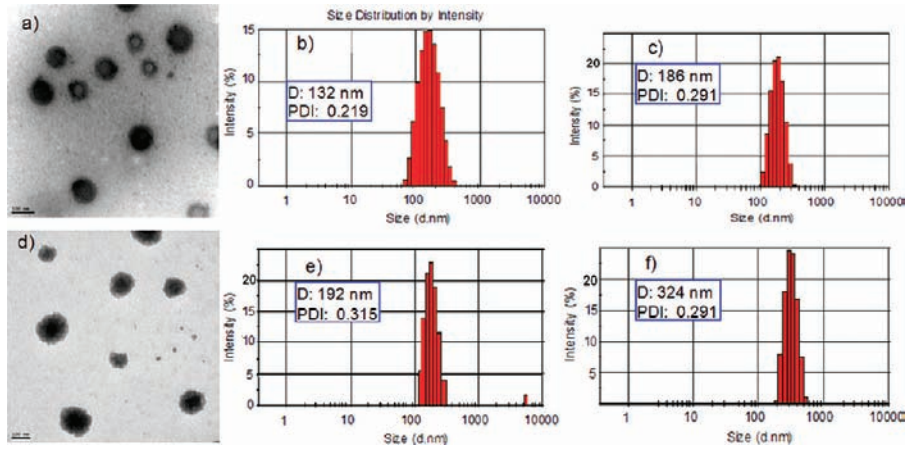


Figure 1. Top panel: OEG-DiCPT-formed liposome-like nanocapsules observed by transmission electron microscopy (TEM) (a, scale bar of 100 nm, dyed with water-insoluble cisplatin), and the nanocapsules measured by dynamic laser light scattering (DLS) before (b) and after (c) loading with 18.5 wt % water-soluble DOX·HCl. Bottom panel: OEG-CPT-formed liposome-like vesicles observed by TEM (d, scale bar of 100 nm, dyed with water-soluble phosphotungstic acid stain), and the nanocapsules measured by DLS before (e) and after (f) loading with 14.2 wt % water-soluble DOX·HCl.

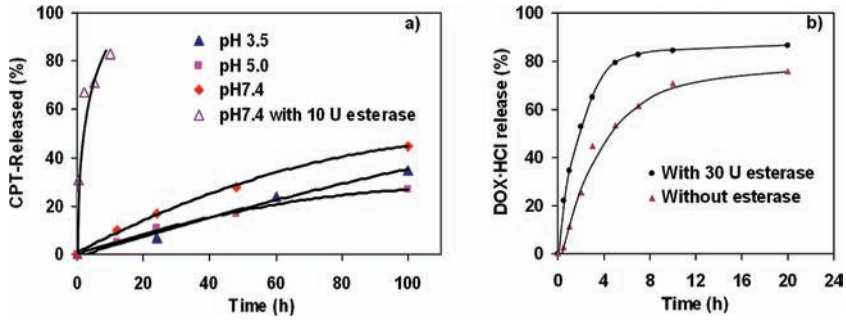


Figure 2. CPT release kinetics from the OEG-DiCPT nanocapsules (a) and the DOX·HCl release kinetics in PBS at pH 7.4 and 37 °C from the DOX·HCl-loaded nanocapsules (esterase concentration, 30 U) (b).

132 nm in diameter (Figure 1b) with a ζ-potential of −4.0 mV. OEG-CPT nanocapsules were about 192 nm in diameter.

The liposome-like structures were further confirmed by encapsulation of water-soluble doxorubicin/hydrogen chloride salt (DOX·HCl). DOX·HCl was loaded into the nanocapsules using a dialysis method similar to drug loading into conventional liposomes. The loading efficiency was surprisingly high, 85%, and the loading content was 18.5 wt % in OEG-DiCPT nanocapsules. The loading efficiency into the OEG-CPT nanocapsules was slightly lower, and its loading content was 14.2 wt %. A similar result was obtained in loading highly water-soluble fluorescent dye calcein (15.5 wt % loading content). The vesicles loaded with DOX·HCl or calcein had bright fluorescent cores clearly seen using confocal laser scanning fluorescence microscopy (Supporting Information Figure S4). As a control, water-soluble DOX·HCl or calcein was found to not be able to be loaded in typical micelles made from polycaprolactone-*block*-polyethylene glycol (PCL(2kDa)-PEG(2kDa)). Thus, loading of water-soluble compounds into the particles further confirmed their vesicular structure. The DOX·HCl-loaded nanocapsules became slightly larger, but were more uniform in size (Figure 1c,f). OEG-DiCPT/DOX·HCl nanocapsules were about 186 nm in diameter, and the OEG-CPT/DOX·HCl nanocapsules became 324 nm. We found that loading of highly water-soluble molecule calcein at a loading content of 15.5 wt % also increased the OEG-DiCPT vesicle size to 176 nm. Therefore, the increase of the vesicle sizes after loaded with DOX·HCl was not due to the insertion of the hydrophobic part of DOX·HCl into the vesicle membrane. The exact cause is still under investigation. The OEG-CPT and its DOX·HCl-loaded nanocapsules were too large for drug delivery, and thus the following study only focused on the OEG-DiCPT nanocapsules.

The OEG-DiCPT nanocapsules contained 58.5 wt % CPT, as calculated from its molecular structure in Scheme 1. This fixed drug loading content is another advantage of the prodrug nanocapsules over conventional nanoparticles and liposomes, whose drug contents may vary from batch to batch. The release of CPT from the OEG-DiCPT nanocapsules was determined using HPLC (Supporting Information Figure S3). OEG-DiCPT very slowly hydrolyzed and released the CPT at a weakly acidic or neutral pH without the common burst release phenomenon found in drug-loaded nanoparticles or liposomes (Figure 2a). However, in the presence of esterase, which is abundant in cytoplasm, OEG-DiCPT quickly hydrolyzed and released the CPT. Therefore, OEG-DiCPT was a prodrug for intracellular release of CPT.

The utility of the OEG-DiCPT nanocapsules as carriers for other drugs was also demonstrated by the controlled release of DOX·HCl (Figure 2b). The OEG-DiCPT/DOX·HCl nanocapsules released DOX·HCl in a controlled manner, suggesting that the water-soluble DOX·HCl had to slowly diffuse out through the hydrophobic nanocapsule membrane made of CPT molecules. Adding esterase promoted DOX·HCl release due to the hydrolysis of OEG-DiCPT.

Intracellular Uptake of the Prodrug Nanocapsules. The ability of DOX·HCl-loaded OEG-DiCPT nanocapsules (OEG-DiCPT/DOX·HCl) to enter cancer cells was observed by confocal laser

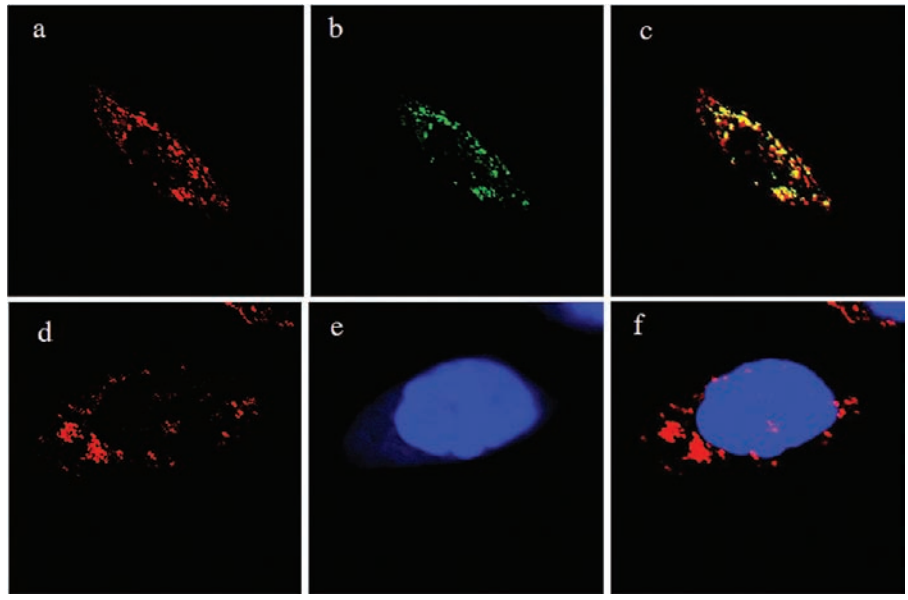


Figure 3. Subcellular localization of OEG-DiCPT/DOX·HCl nanocapsules observed by confocal fluorescent microscopy. SKOV-3 ovarian cancer cells were incubated with OEG-DiCPT/DOX·HCl at a DOX dose of 1 μg/mL for 5 h at 37 °C. Top panel: Observation of the lysosomal localization from the DOX channel (a), LysoTracker green (DND-26, to label lysosomes) channel (b), and the overlap of the (a) and (b) images (c). Bottom panel: Observation of the nuclear localization from the DOX channel (d), the nuclear dye DRAQ5 channel (e), and the overlap of the (d) and (e) images (f) (larger view images are shown in Figure S5 in Supporting Information).

scanning microscopy (Figure 3). SKOV-3 ovarian cancer cells were cultured with OEG-DiCPT/DOX·HCl nanocapsules for 5 h before examination. DOX·HCl is fluorescent and was used as a dye to label the nanocapsules. Lysosomes were labeled with LysoTracker. As shown in Figure 3, OEG-DiCPT/DOX·HCl was quickly taken up and mostly localized in the lysosomes (yellow spots in Figure 3c). Some nanocapsules not associated with lysosomes (red spots in Figure 3c) were probably those that had already escaped from the lysosomes within the 5 h incubation. After the nuclei of the tumor cells were stained with DRAQ5 (blue in Figure 3e,f), many OEG-DiCPT/DOX·HCl nanocapsules were found to be associated with or near the nuclear membrane, and some were even located in the nuclei (pink spots in Figure 3f; see the larger view images in the Supporting Information Figure S5). Free DOX·HCl can easily enter the non-ADR SKOV-3 cancer cells, accumulate, and homogeneously distribute in the nuclei.[21] Thus, the punctuated distribution of OEG-DiCPT/DOX·HCl fluorescence in the cytoplasm and the nuclei indicates that DOX·HCl entered the tumor cells by the delivery of OEG-DiCPT nanocapsules rather than by diffusion of the free drug.

OEG-DiCPT as a Prodrug Releasing Active CPT. Figure 2 shows that OEG-DiCPT is a prodrug releasing CPT. The ability of OEG-DiCPT to act as a CPT prodrug was further confirmed by its induced cancer cell apoptosis observed using the DAPI staining (Figure 4a−d). SKOV-3 ovarian cancer cells were cultured with OEG-DiCPT nanocapsules at CPT equivalent doses of 0.5 and 2 μg/mL for 72 h. The cell number was substantially lower after the treatment, and the nuclei of the remaining cells shrank to some extent compared with the cells without treatment (b and c versus a of Figure 4). This result suggests that OEG-DiCPT inhibited tumor proliferation and induced apoptotic cell death. Further, Z-DEVD-FMK, a caspase-3 inhibitor, was used to examine the possible role of caspase-3 in OEG-DiCPT-induced apoptosis. Pretreatment with Z-DEVD-FMK blocked some OEG-DiCPT-induced apoptosis (d versus c in Figure 4), indicating that caspase-3 activation may play a role in mediating OEG-DiCPT-induced apoptosis.

BrdU can be incorporated into newly synthesized DNA of replicating cells. Therefore, we performed a BrdU incorporation assay to determine the potential effect of OEG-DiCPT on DNA synthesis in SKOV-3 cells.[22] In brief, BrdU was labeled with DyLight 549 (red in Figure 4e−h), and the cell nuclei were stained by DAPI (blue in Figure 4i−l). One hundred cells were counted randomly for each group to calculate the percentage of BrdU-stained cells. The data showed that around 25.1% of the tumor cells in the control group exhibited BrdU immunofluorescence. However, after the cells were treated with OEG-DiCPT at doses of 0.5, 1, or 2 μg/mL, the proportion of BrdU-stained cells decreased to 20.2, 15.4, and 5.3%, respectively. Meanwhile, the BrdU fluorescence intensity decreased as the dose of OEG-DiCPT increased (f−h versus e in Figure 4). These results suggest that OEG-DiCPT significantly suppressed DNA synthesis and subsequently inhibited cell proliferation. To confirm this result, several cell cycle-related proteins, including Cyclin D1, CDK4, and CDK6, were examined. Cyclin D1 is known to bind to CDK4 and CDK6 to form a pRb kinase, and these proteins play essential roles in the transition from the G1/G0- to S-phase and for DNA replication.[23] All three of these proteins were significantly down-regulated after treatment with OEG-DiCPT (Figure 5), providing additional evidence for the inhibitory effect of OEG-DiCPT on DNA synthesis. In addition, OEG-DiCPT significantly down-regulated the protein level of pro-caspase-3 in SKOV-3 cells (Figure 5, panel 4), which is consistent with the above data that caspase-3 activation is involved in OEG-DiCPT-induced apoptosis.

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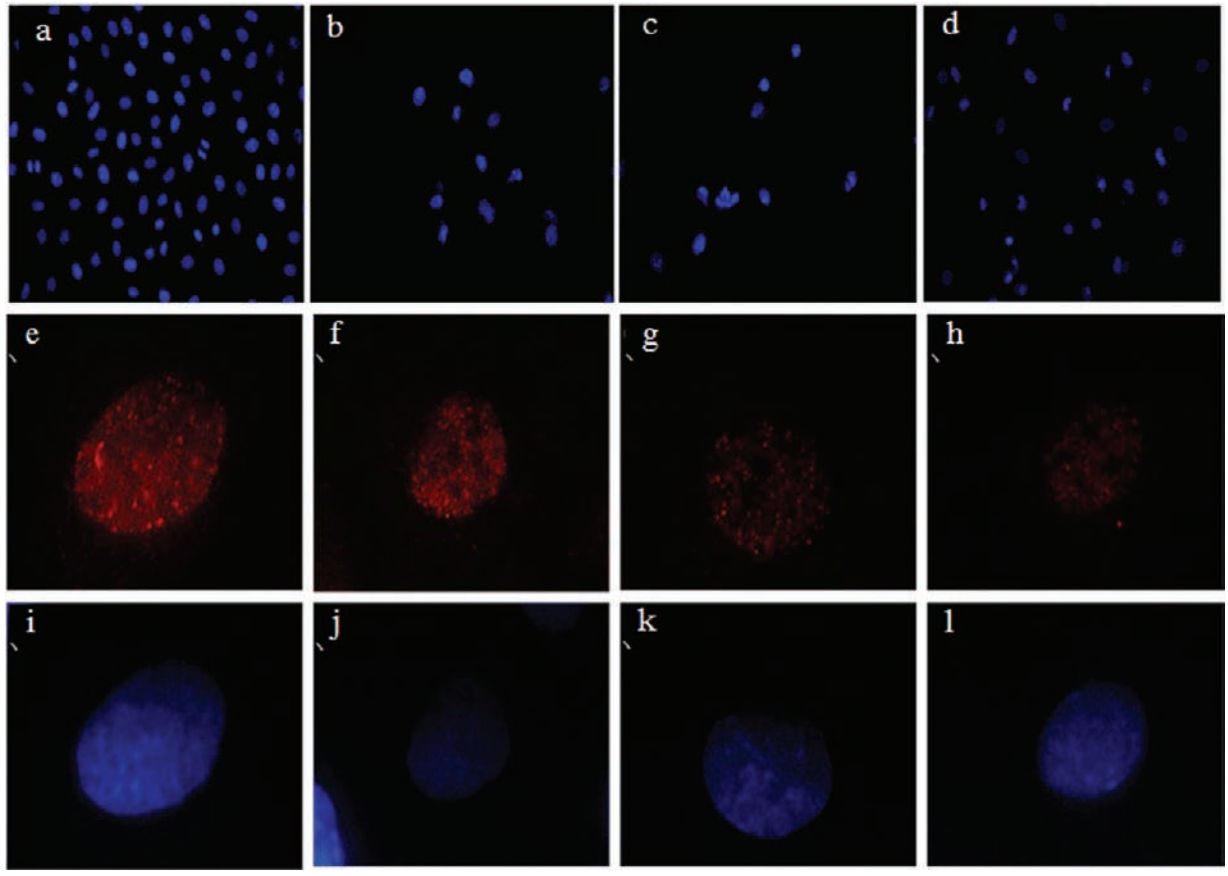

Figure 4. OEG-DiCPT-induced apoptosis of SKOV-3 cells detected by DAPI staining (a−d) and the suppression of DNA synthesis detected by BrdU incorporation (e−l). Top panel (a−d): Cells were untreated (a) or treated with 0.5 μg/mL (b) or 2 μg/mL (c) of OEG-DiCPT for 72 h before adding DAPI for observation. In (d), the cells were pretreated with 100 μM DEVD-FMK for 2 h followed by adding 2 μg/mL of OEG-DiCPT and incubation for 72 h. Original magnification 20×. Middle (e−h, BrdU stained cell nucleus) and bottom (i−l, DAPI stained cell nucleus) panels: SKOV-3 cells were untreated (e,i) as the control or treated with OEG-DiCPT at 0.5 μg/mL (f,j), 1 μg/mL (g,k), or 2 μg/mL (h,l) for 24 h before BrdU staining (red, labeled DyLight 549-conjugated secondary antibody) or DAPI staining (blue) as described in the Supporting Information. Original magnification 100×. Three independent experiments yielded equivalent results for both assays.

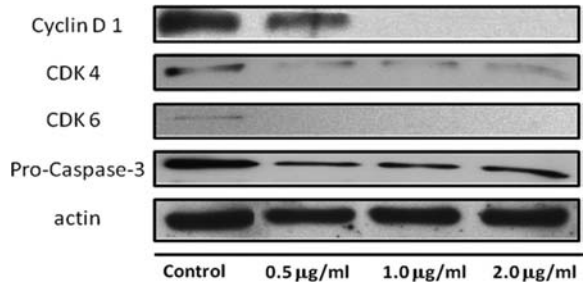

Figure 5. Protein expression detected by Western Blot. Cells were left untreated (control) or treated with 0.5, 1, or 2 μg/mL of OEG-DiCPT for 24 h before protein extraction. Equal amounts of proteins were fractionated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane, followed by immunoblotting with anti-Cyclin D1, CDK4, CDK6, and pro-caspase-3 antibodies. β-Actin was used for normalization of protein loading. Three independent experiments yielded equivalent results.

In Vitro and In Vivo Anticancer Activities. The overall cytotoxicity of OEG-DiCPT was evaluated and compared with CPT as a free drug using MTT assays in SKOV-3 ovarian and MCF-7 breast cancer cell lines. The OEG-DiCPT nanocapsules loaded with DOX·HCl were also evaluated under identical conditions. As depicted in Figure 6, OEG-DiCPT showed similar cytotoxicity to both SKOV-3 and MCF-7 cancer cells when compared with free CPT. In conjunction with the above results, we can conclude that OEG-DiCPT is a prodrug, which releases active CPT once inside cancer cells inducing their apoptosis.

OEG-DiCPT/DOX·HCl showed a higher cytotoxicity than OEG-DiCPT, free CPT, and free DOX in all the doses tested in MCF-7 cells, as well as in most doses tested in the SKOV-3 cells. This finding is in agreement with the above results showing that OEG-DiCPT-formed nanocapsules served as a carrier to deliver DOX·HCl into the cells, producing additive or synergistic anticancer effects.

The in vivo antitumor activity of OEG-DiCPT was preliminarily assessed using athymic mice bearing intraperitoneal (ip) tumors (Figure 7). One intravenous (iv) injection at a dose of 5 mg/kg to each mouse significantly reduced the ip tumor burden to 1/4 of the control. This fast and significant tumor suppression was comparable to the CPT conjugate having BH3 targeting groups,[24] indicating that OEG-DiCPT had a strong in vivo anticancer activity. The high antitumor efficacy may suggest that OEG-DiCPT nanocapsules could target tumor tissues via tumors' (EPR) effect[12] due to their optimal particle size (around 100 nm). In contrast, CPT itself is difficult for iv administration

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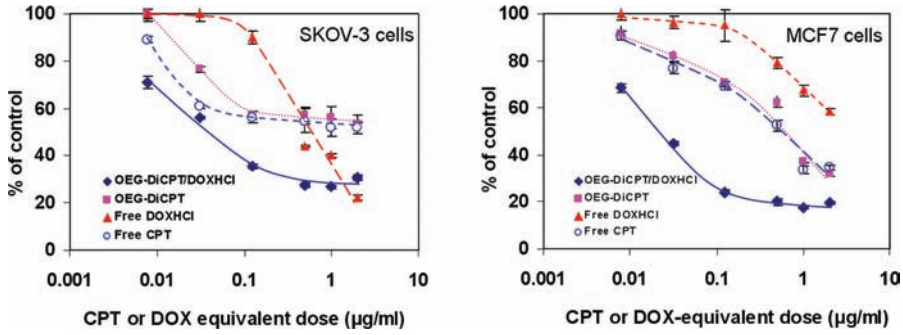

Figure 6. In vitro cytotoxicity of CPT, OEG-DiCPT, DOX·HCl, and OEG-DiCPT/DOX·HCl to SKOV-3 ovarian cancer cells and MCF-7 breast cancer cells determined by MTT assay. Cells were cultured with the treatments for 24 h followed by a 24 h postculture.

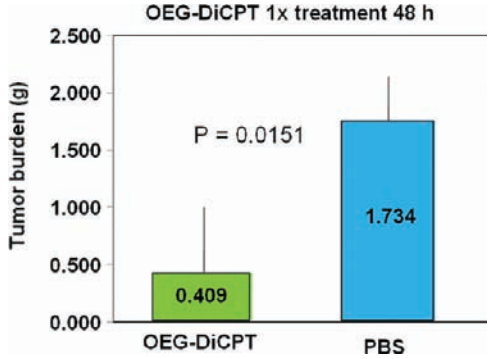

Figure 7. In vivo antitumor activity of OEG-DiCPT to xenografted intraperitoneal SKOV-3 ovarian tumors in BALB/c strain nu/nu mice; 0.1 mL of OEG-DICPT (CPT equivalent, 1 mg/mL in PBS) solution was administered via the tail vein at 4 weeks post-inoculation (when tumors along the mesentery were well-established). Tumors were dissected at necropsy 48 h following the treatments. Cumulative wet tumor weights per mouse were determined.

due to its water insolubility. The encouraging data merit further detailed in vivo study.

Conclusion

In conclusion, we demonstrated a new concept of directly using drug molecules as components of nanocarriers to substantially increase the drug loading content, minimize use of inactive materials, and suppress premature burst release of the drug. With hydrophobic CPT as the hydrophobic tails and a short OEG chain as the hydrophilic head, amphiphilic phospholipid-mimicking prodrugs were prepared. The OEG-DiCPT formed stable liposome-like nanocapsules with a CPT loading content as high as 58 wt % without burst release in aqueous solution. OEG-DiCPT itself was a prodrug, releasing CPT once inside cells that showed high in vitro and in vivo antitumor activity. Meanwhile, the formed nanocapsules could be loaded with water-soluble drugs such as DOX·HCl with a high loading efficiency. The DOX·HCl-loaded nanocapsules delivered two anticancer drugs, producing a synergetic cytotoxicity to cancer cells. This prodrug nanocapsule is a new efficient drug carrier for cancer chemotherapy. Detailed in vivo study including the prodrug's in vivo toxicity, pharmacokinetics, biodistribution, and antitumor activity nanocapsules is currently underway.

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Supporting Information Available: Full data of synthesis methods and characterizations of the compounds using NMR, preparation of the nanocapsules and drug loading, drug release, cellular uptake, apoptotic cell death, and DNA synthesis assays, Western blotting, cytotoxicity, and in vivo anticancer activity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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