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Research paper

Comparative evaluation of polymeric and amphiphilic cyclodextrin nanoparticles for effective camptothecin delivery

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ABSTRACT

Camptothecin (CPT) is a potent anticancer agent. The clinical application of CPT is restricted by poor water solubility and instability under physiological conditions. Solubilization and stabilization of CPT were realized through nanoparticulate systems of amphiphilic cyclodextrins, poly(lactide-co-glycolide) (PLGA) or poly-ε-caprolactone (PCL). Nanoparticles were prepared with nanoprecipitation technique, whereas cyclodextrin nanoparticles were prepared from preformed inclusion complexes of CPT with amphiphilic cyclodextrins. Polymeric nanoparticles, on the other hand, were loaded with CPT:HP-β-CD inclusion complex to solubilize and stabilize the drug. Mean particle sizes were under 275 nm, and polydispersity indices were lower than 0.2 for all formulations. Drug-loading values were significantly higher for amphiphilic cyclodextrin nanoparticles when compared with those for PLGA and PCL nanoparticles. Nanoparticle formulations showed a significant controlled release profile extended up to 12 days for amphiphilic cyclodextrin nanoparticles and 48 h for polymeric nanoparticles. Anticancer efficacy of the nanoparticles was evaluated in comparison with CPT solution in dimethyl sulfoxide (DMSO) on MCF-7 breast adenocarcinoma cells. Amphiphilic cyclodextrin nanoparticles showed higher anticancer efficacy than PLGA or PCL nanoparticles loaded with CPT and the CPT solution in DMSO. These results indicated that CPT-loaded amphiphilic cyclodextrin nanoparticles might provide a promising carrier system for the effective delivery of this anticancer drug having bioavailability problems.

1. Introduction

Camptothecin (CPT), a plant alkaloid isolated from an oriental tree, *Camptotheca acuminata*, was first identified in the 1950s [1]. CPT has shown significant antitumor activity against various cancers, including lung, ovarian, breast, pancreas and stomach, by inhibiting the activity of DNA topoisomerase I, which is required for replication and transcription of the cell cycle [2–4]. DNA topoisomerase I is believed to stabilize the DNA-topoisomerase complex, and this complex causes the apoptosis of cancer cells [5–7].

However, clinical efficacy of CPT is significantly restricted by its insolubility and instability. The drug exists in two forms depending on the pH value, an active lactone form at pH below 5 and an inactive carboxylate form at basic pH, as schematized in Fig. 1. At physiological pH, most CPT molecules exist in the inactive carboxylate form. Development of a formulation capable of maintaining the stable lactone form of CPT is crucial for the achievement of clinical efficacy [8–10]. Another serious drawback of CPT is its water insolubility. CPT is soluble only in dimethylsulfoxide (DMSO), dichloromethane:methanol (1:1) (v:v) and chloroform:methanol (4:1) (v:v). Thus, CPT, like a number of other potent anticancer agents of plant origin such as paclitaxel, is extremely water insoluble [6], and this leads to the usage of co-solvents that exert serious side effects upon injection and causes bioavailability problems for CPT when administered in vivo.

Numerous studies have focused on designing novel drug delivery systems for CPT to stabilize and deliver the drug effectively for clinical use [11,12]. In this respect, liposomes, microspheres, solid lipid nanoparticles, polymeric nanoparticles (PLGA, PLA/(PEG–PPG–PEG), chitosan), microemulsions, micellar solutions, and polymer-conjugated CPT were proposed [13–18].

Cyclodextrins have lipophilic inner cavities and hydrophilic outer surfaces. They are capable of interacting with a large variety of guest molecules to form noncovalent inclusion complexes. Cyclodextrins are cyclic oligosaccharides and contain at least six D-(+) glucopyranose units which are attached by α-(1, 4) glucosidic bonds. They have been widely used for the formulation of drugs having bioavailability problems resulting from poor aqueous solubility, poor stability (hydrolytic or photodegradation, etc.) and severe side effects [19–21]. Amphiphilic cyclodextrins are synthetic derivatives of natural cyclodextrins, obtained by modification of

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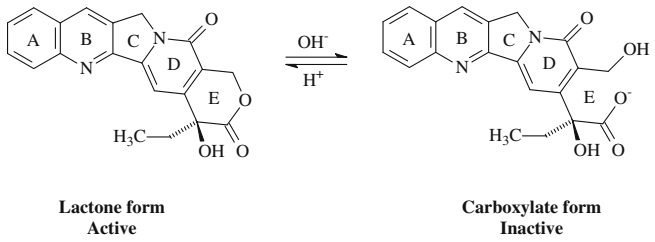

Fig. 1. Camptothecin structure and equilibrium between active lactone form and inactive carboxylate form.

the primary or secondary phase with linear or branched aliphatic chains of varying lengths (C2–C18) linked with different chemical bonds (ester, ether or amide) [22]. These derivatives are of interest for pharmaceutical applications in view of their ability to self-organize in water to form micelles and nano-aggregates, which is a result of their altered surface properties and their general amphiphilic characteristics. Amphiphilic cyclodextrins have recently been used to prepare nanoparticles and nanocapsules without surfactants and have shown high drug-loading capacity with favorable release properties. Among the amphiphilic cyclodextrin derivatives used in this study, 6-O-Capro-β-CD is a novel amphiphilic β-CD derivative modified on the primary phase with substitution of C6 linear alkyl chains and hydrophobic ester bond [23]. β-CDC6, on the other hand, is an amphiphilic β-cyclodextrin modified on the secondary phase with 6C aliphatic esters [24]. Different modes of substitution are believed to alter inclusion-forming properties and nanoparticle-forming capability and inclusion complex formation of amphiphilic cyclodextrin derivatives [25]. Therefore, amphiphilic β-CD derivatives with different substitutions on primary or secondary faces were evaluated for a comparative assessment within amphiphilic cyclodextrin nanoparticles.

Poly(lactide-co-glycolide) (PLGA) is the most widely used and studied class of biodegradable polymers for pharmaceutical use due to biocompatibility and biodegradability [16]. The degradation of PLGA proceeds with the formation of low-molecular-weight carboxylic acid oligomers and monomers and it causes a decrease in microclimate pH. The pH is reported to strongly affect CPT stability. The acidic microclimate of PLGA nanospheres has been identified to be the important source of stabilization of CPT [10]. PLGA microspheres have been studied as delivery vehicles for CPT and its analogues because PLGA microspheres have been considered stable delivery vehicles for CPT [9]. Poly-ε-caprolactone (PCL) is a biodegradable, biocompatible and semicrystalline polymer. The slow degradation of PCL has led to its application in the preparation of different delivery systems in the form of microspheres, nanospheres and implants [26].

The objective of this study was to develop, characterize, and compare in vitro nanoparticulate drug delivery systems for CPT using amphiphilic β-cyclodextrins, PLGA or PCL. CPT was encapsulated directly into two different types of amphiphilic β-cyclodextrins, namely 6-O-Capro-β-CD and β-CDC6. CPT was incorporated into PLGA or PCL nanoparticles in complex with hydroxypropyl-β-cyclodextrin in order to improve drug solubility and stability. All nanoparticle formulations were comparatively evaluated in terms of particle size, entrapment efficiency, drug release profiles, cytotoxicity of blank vehicles and anticancer efficacy of CPT-loaded nanoparticulate vehicles with cell culture studies.

2. Materials and methods

2.1. Materials

20S-Camptothecin (CPT, MW: 348.36 g/mol, soluble in dimethylsulfoxide, dichloromethane:methanol (1:1) and chloroform:methanol (4:1)) was purchased from Sigma Aldrich (St. Louis, USA). Amphiphilic β-cyclodextrin derivative β-CDC6 (MW: 2506 g/mol, calculated HLB value: 8.9), modified on the secondary phase, and 6-O-Capro-β-CD (MW: 1820 g/mol, calculated HLB value: 11.1), modified on the primary phase with 6C aliphatic chains, were synthesized, purified and characterized as reported previously [27] (Table 1). (2-Hydroxypropyl)-β-cyclodextrin (HP-β-CD) (MW: 1396 g/mol), poly-ε-caprolactone (PCL) (MW: 14,000 Da) and Poly(lactide-co-glycolide) (PLGA 50:50) (MW: 40,000–75,000 Da) were purchased from Sigma Aldrich (St. Louis, USA). Pluronic F68 was obtained from ICI Surfactants (Clamart, France). Acetone was purchased from Riedel-de Haen (Seelze, Germany). Absolute alcohol was purchased from Grup Deltalar (Ankara, Turkey). Tween 80 was obtained from Merck-Schuchardt (Hohenbrunn, Germany). Ultra pure water (Milipore Simplicity 185, France) was used to prepare nanoparticles. All organic solvents were of high-performance liquid chromatography (HPLC) grade and were used without further purification.

2.2. Methods

2.2.1. Preparation of inclusion complexes

2.2.1.1. CPT:β-CDC6 and CPT:6-O-Capro-β-CD complexes. CPT:β-CDC6 and CPT:6-O-Capro-β-CD inclusion complexes of 1:1 molar ratio were prepared in a water/ethanol system by co-lyophilization technique [28]. CPT and the amphiphilic cyclodextrin (β-CDC6 or 6-O-Capro-β-CD) were dissolved in an appropriate molar ratio in ethanol and added to a 2-fold volume of water. The system was left to equilibrate under constant stirring for 7 days at room temperature. The organic solvent was completely evaporated under vacuum (Buchi R-3000, Switzerland), and the aqueous suspension was lyophilized (Heto Power Dry PL 3000, Denmark) and stored as powder.

2.2.1.2. CPT:HP-β-CD complex. The inclusion complex of CPT with HP-β-CD in a 1:1 molar ratio was prepared according to the co-lyophilization technique [28]. CPT and cyclodextrin were dissolved in an appropriate molar ratio in water. The system was left to equilibrate under constant stirring for 7 days at room temperature. At the end of equilibrium time, the dispersion was filtered, and the filtrate containing soluble CPT:dHP-β-CD complex (1:1) was lyophilized using a Heto-Holten A/S Lyolab Freeze Dryer (Allerod, Denmark) to obtain the complex in dry powder form.

2.2.2. Characterization of inclusion complexes

2.2.2.1. Thermal analysis. Differential scanning calorimetry (DSC) was used to evaluate physicochemical state of CPT within 1:1 molar ratio (drug:CD) complexes. DSC thermograms were taken with a DuPont DSC 910 Instrument (DuPont, USA) in a temperature range of 25–350 °C under nitrogen atmosphere. Samples were heated in hermetically sealed aluminium pans at a rate of 10 °C/min.

Table 1
Physicochemical properties of amphiphilic cyclodextrins and polymers used for preparing nanoparticle formulations.

	6-O-Capro-β-CD	β-CDC6	PLGA	PCL
Molecular weight	1820 g/mol	2506 g/mol	40,000–75,000 Da	14,000 Da
Solubility	Alcohol, acetone	Alcohol, acetone	Chlorinated solvents, tetrahydrofuran, acetone, ethyl acetate.	Acetone
Spontaneous formation of nanoparticles	Non-surfactant	Non-surfactant	Surfactant required	Surfactant required

2.2.2.2. ^1H NMR spectroscopy. Proton nuclear magnetic resonance (^1H NMR) spectra of CPT, 6-O-Capro-β-CD, β-CDC6 and CPT:cyclodextrin complexes were taken by a Bruker DPX 400 digital FT-NMR spectrophotometer (Rheinstetten, Germany) at 400 MHz. Chemical shifts were given to external tetramethylsilane at 0 ppm with calibration using solvent signals (dimethylsulfoxide at 2.5 ppm, deuterated water at 4.75 ppm, and chloroform $CHCL_3$ at 7.25 ppm).

2.2.3. Preparation of CPT-loaded amphiphilic β-cyclodextrin nanoparticles

Nanoparticles were prepared according to the nanoprecipitation method introduced by Fessi et al. [29] and further modified [25] to prepare the nanoparticles directly from preformed inclusion complexes of CPT and cyclodextrins. The organic phase (0.8 mL) consisting of 1 mg of CPT:amphiphilic β-CD inclusion complex dissolved in absolute alcohol was prepared, and 0.2 mL of CPT solution in absolute alcohol was added to this organic phase. This solution was added at room temperature under constant stirring to 2 mL of the aqueous phase consisting only of ultrapure water. After stirring for 30 min at room temperature, the organic solvent was evaporated under vacuum, and the nanoparticle dispersion was concentrated to the desired volume (2 mL). This technique was called high-loading method. Highly loaded nanospheres were prepared directly from preformed drug:cyclodextrin inclusion complexes and by further dissolving an additional amount of drug (200 μg) in the organic phase during preparation [24].

2.2.4. Preparation of CPT-loaded polymeric nanoparticles

The nanoparticles were prepared by nanoprecipitation method [30,31]. Briefly, 75 mg of polymer (PLGA or PCL) was dissolved in 5 mL of acetone. This organic phase was poured into deionized water (15 mL for PLGA, 10 mL for PCL nanoparticles) containing 75 mg of Pluronic F-68 and 2,5 mg CPT:HP-β-CD inclusion complex with moderate stirring at room temperature. Nanoparticles were immediately formed, and acetone was then removed from the colloidal suspension by rotoevaporation under reduced pressure, and nanosphere dispersion was obtained.

2.2.5. Characterization of nanoparticles

2.2.5.1. Particle size analysis. Mean particle size (diameter, nm ± S.D.) and polydispersity index of the nanoparticles were determined by Photon Correlation Spectroscopy (PCS) with a Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK). Measurements were realized in triplicate at a 90° angle at 25 °C under suitable dilution conditions.

2.2.5.2. Zeta potential measurement. Zeta potential of nanoparticle dispersions was measured in mV by Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) in triplicate to determine the surface charge and the potential physical stability of the nanosystem. Zeta potential of nanoparticles was measured in aqueous dispersion. Measurements were realized in triplicate at a 120° angle at 25 °C.

2.2.5.3. Scanning electron microscope analysis. A scanning electron microscope (SEM) (Jeol-SEM ASID-10. Device in 80 KV, Japan) was used to evaluate surface characteristics of the nanoparticles. Nanoparticles were mounted on the metal stubs with conductive silver paint and then coated with a 150 Å thick layer of gold in a Bio-Rad sputter apparatus. SEM images of the samples were obtained at different magnifications.

2.2.5.4. Entrapment efficiency. Loaded drug quantity was determined according to the following procedure: unbound drug was separated by centrifugation (Hermle Z-323 K, Germany) at 5000 rpm for 15 min. Supernatant was then collected and lyophilized, and the resulting powder containing the loaded nanoparticles was dissolved in dimethylsulfoxide to obtain a clear solution and analyzed by HPLC (Agilent 1100, Germany).

Drug-loading values were expressed in terms of entrapped drug quantity, entrapment efficiency and associated drug percentage. Entrapped drug quantity is the exact drug concentration (μg) found per nanoparticle batch. The following formulas were used to calculate entrapment efficiency and associated drug percentage, respectively [32]:

$$\text{Associated drug } \% = \frac{\text{Determined CPT quantity}(\mu g)}{\text{Initial CPT quantity}(\mu g)} \times 100$$

2.2.5.5. HPLC assay for camptothecin. The analyses of lactone and carboxylate forms of CPT were performed using HP Agilent 1100, USA. The system was equipped with a C18 column (5 mm, 250 × 4.6 mm, Hichrom, UK). The HPLC conditions used were similar to those reported by Warner and Burke [33], except for a minor change in the mobile phase. The mobile phase consisted of a 73:27 mixture of aqueous triethylamine-acetate buffer (prepared using 0.1% v/v triethylamine, adjusted with glacial acetic acid to pH 5.5) and acetonitrile. The fluorescence detector was set at an excitation wavelength (λ_{ex}) of 370 nm and an emission wavelength (λ_{em}) of 435 nm. Validation of the method used was realized by the determination of linearity, accuracy, precision, sensitivity and specificity (r^2 = 0.9999; $Rt_{lactone}$ = 13 min, $Rt_{carboxylate}$ = 4 min).

2.2.5.6. Determination of hydrolysis kinetics for CPT. Hydrolysis kinetics of the free drug in phosphate-buffered saline, (PBS) pH 7.4 was also evaluated over time at 37 °C to determine the rate of degradation of CPT under physiological pH [16]. For this purpose, 5 μL of CPT stock solution (2 mM CPT in DMSO) was added to 500 μL PBS, pH 7.4. At different time intervals, aliquots of the reaction solutions were withdrawn, injected directly into the HPLC column, and analyzed for the amount of lactone and carboxylate forms. The stability of the lactone form of CPT was represented by the percentage of the lactone form compared with that of the initial time (t = 0). All experiments were performed in triplicate.

2.2.5.7. In vitro drug release. Release profiles of CPT from nanoparticle formulations were determined in 100 mL of isotonic PBS (pH 7.4) containing 0.1% Tween 80 providing sink conditions in a thermostated shaker bath system (Memmert, Schwabach, Germany) at 37 °C with the dialysis technique (Spectra/Por Cellulose Ester Membrane MWCO:100,000 Da, Spectrum Labs, Rancho Dominguez, CA). At predetermined time intervals, 1 mL samples were withdrawn from the system and replaced with equal volume of fresh release medium maintained at the same temperature. The released amount of CPT was assayed for lactone and carboxylate forms by HPLC as described above.

2.2.6. Cell culture

L-929, mouse fibroblast and MCF-7 human breast adenocarcinoma cell lines were obtained from the American Type Culture Collection (ATCC, LGC Promochem, Rockville, MD, USA). L-929 and MCF-7 cell lines were cultured in RPMI 1640 medium and Dulbecco's Modified Eagle Medium (DMEM), respectively, supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL). The cultures were maintained at 37 °C in a humidified 5% CO_2 incubator. Confluent cell monolayers were trypsinized, and cells in the exponentially growing phase were used in the cytotoxicity experiments. Unless otherwise specified, all reagents were obtained from Biochrom (Berlin, Germany).

2.2.6.1. The effect of unloaded nanoparticles on L929 Cells. The methylthiazolyltetrazolium (MTT) assay was used to evaluate cell

viability [34]. The MTT assay measures the ability of viable cells to reduce a water-soluble, yellow tetrazolium salt (MTT) into a purple, insoluble formazan product. The color reaction is used as a measure of cell viability. L929 cells were resuspended in complete medium and seeded in 96-well tissue culture plates at a concentration of 2×10^3/50 μl per well and then exposed to 50 μl of diluted formulations ranging from 1:8 to 1:128. After 72 h of incubation, 25 μl of MTT solution (5 mg/mL; Sigma, USA) was added. The formazan crystals produced were solubilized by adding 80 μl of lysing buffer (pH 4.7) composed of 23% sodium dodecyl sulfate (SDS) dissolved in a solution containing 45% dimethylformamide. Optical densities (ODs) were read at 570 nm using a microplate reader (Molecular Devices, USA). The cells incubated in culture medium alone served as a control for cell viability (untreated cells). All assays were performed in triplicate, and mean OD values were used to estimate the cell viability. Cell viability (%) was calculated as (OD of treated wells/OD of untreated cells) × 100.

2.2.6.2. Anticancer efficacy of CPT-loaded nanoparticles on MCF-7 cells. The cytotoxicity of CPT-loaded nanoparticles, unloaded nanoparticles and CPT solution in DMSO was assessed on MCF-7 cells with MTT assay. MCF-7 cells were seeded in 96-well tissue culture plates at a concentration of 1×10^4/50 μl per well. The cells were allowed to attach to the surface for 24 h, and were then exposed to 50 μl of diluted formulations ranging from 1:8 to 1:128. After 72 h of incubation, MTT assay was performed just as described above.

3. Results and discussion

In this study, delivery systems of different nanosizes for the anticancer drug CPT were designed and developed. In this context, two approaches for the stabilization of CPT have been combined. Inclusion complex formation with cyclodextrin derivatives and nanoparticle encapsulation have been used to deliver CPT in its stable form.

In particular, the use of cyclodextrin inclusion complex has been shown to resolve solubility and stability problems to improve pharmacokinetic profiles of CPT. In literature to increase the solubility of drug, CPT was solubilized in an organic solvent and then incorporated into the sucrose aspartate surfactant micelle. The micelles were then incorporated into the layers of cationic Mg–Al layered double hydroxides. Finally, the CPT is twice protected from the physiological environment, first by the organic environment of the micelle and second by the durability of the LDH [17].

Inclusion complexes of CPT with HP-β-CD, β-CDC6 and 6-O-Capro-β-CD were characterized by DSC, ^{1}H NMR and SEM. DSC analyses indicate that free crystalline drug did not exist in any complexes. DSC thermograms of CPT, cyclodextrins and CPT:cyclodextrin complexes are seen in Fig. 2. Melting endotherms of CPT that appear around 264–267 °C disappear totally in the CPT:β-CDC6 complex. This may be the result of inclusion of CPT within the CD cavity, as well as entanglement within the "skirt" region comprised of long acyl chains.

In order to rule out the fact that the lyophilization process may alter the crystalline properties of CPT and consequently hide the melting endotherm, CPT was subjected to the co-lyophilization procedure by dissolving in ethanol and mixing with 2-fold volume of distilled water, stirring and vacuum evaporation of the solvent followed by lyophilization to obtain CPT in dry powder form itself during preparation of inclusion complex prior to DSC analysis. DSC thermograms in Fig. 2 also show that the melting and decomposition endotherm of CPT has completely disappeared in the physical mixture as well as in the co-lyophilizate. This may suggest that CPT does not exist in free crystalline form within the complex. The same results were obtained for CPT:β-CDC6 and CPT:HP-β-CD complexes.

^{1}H NMR is one of the most selective tools for the characterization of inclusion complexes and for the demonstration of total or partial inclusion in the cyclodextrin cavity that occurs in a liquid medium. In the case of cyclodextrins, it is important to demonstrate whether the drug is included in the cavity or entrapped within the chains of the molecule. During complexation, the chemical environment of some protons changes, and these results in changes in chemical shifts of ^{1}H NMR lines of the protons that are due to shielding or deshielding effects. For the complexes, internal protons of the cavity, which were H-3 and H-5, were evaluated for changes in ^{1}H NMR spectra. H-5 shifted from 3.86 to

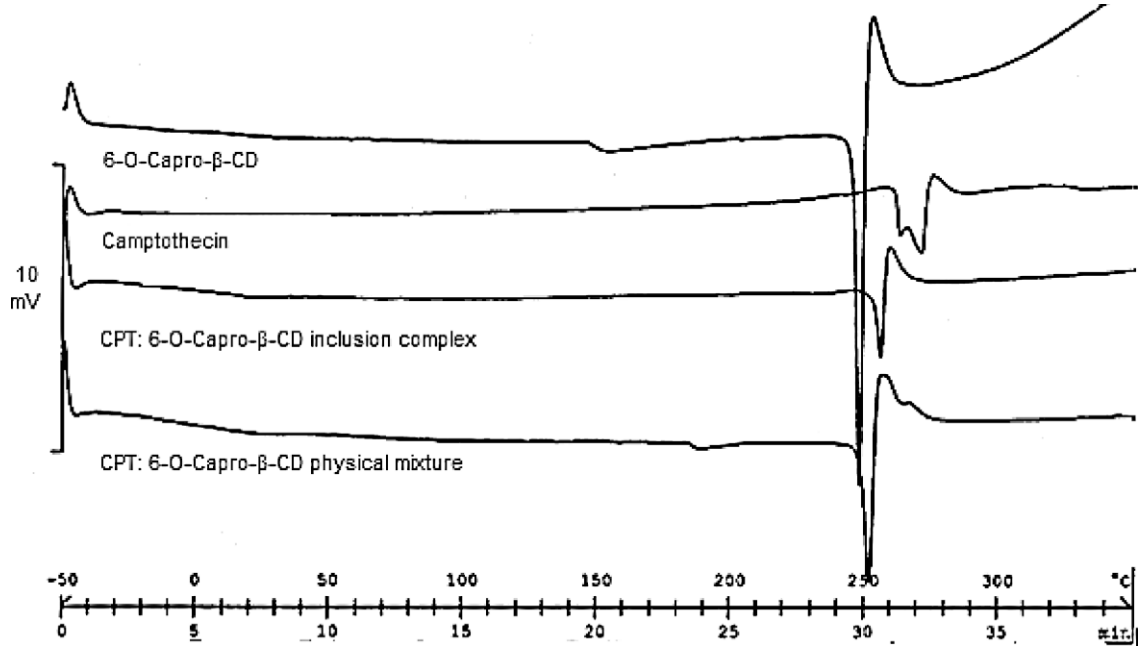

Fig. 2. DSC thermograms of camptothecin, cyclodextrin, inclusion complex and physical mixture.

Table 2
Chemical shift values (ppm) of 6-O-Capro-β-CD and β-CDC6 protons before (δ_{CD}) and after ($\delta_{1:1}$) complexation to CPT.

	Protons	δ_{CD}	$\delta_{1:1}$
6-O-Capro-β-CD	H-3	3.66	3.63
	H-5	3.86	3.88
β-CDC6	H-3	5.30	5.30
	H-5	4.10	4.60

3.88 ppm and H-3 shifted from 3.66 to 3.63 ppm for CPT:6-O-Capro-β-CD; for CPT:β-CDC6, H-5 shifted from 4.10 to 4.6 ppm but H-3 did not display any shifts. These results suggest that the drug has entered the cyclodextrin cavity to form an inclusion complex [23]. For CPT:HP-β-CD complex, internal protons H-3 and H-5 did not show any changes in the NMR spectra (Table 2).

SEM photomicrographs of CPT, cyclodextrins and co-lyophilizates confirmed the existence of structural changes in CPT attributed to complex formation.

Nanoparticles prepared from inclusion complexes were characterized in vitro by particle size measurements. As seen in Table 3, blank nanoparticles were smaller than drug-loaded nanoparticles, which can be attributed to the encapsulation of the drug to the nanoparticle. This may be a result of the inclusion of encapsulated drug in cyclodextrin cavity and moreover between the alkyl chains of each cyclodextrin, which is a zone of attraction for the hydrophobic guest molecule. Once a drug molecule is included inside an amphiphilic cyclodextrin, the orientation and flexibility of long alkyl chains are altered [35]. In the presence of a guest molecule, surface area of the amphiphilic cyclodextrin increases, and this affects the self-aligning property of the amphiphilic cyclodextrin in interfaces. Thus, when nanoparticles are drug loaded, the state and location of the encapsulated drug may cause significant changes in nanoparticle size [23].

In terms of mean diameter, β-CDC6 nanoparticles yielded the smallest particles with approximately 140 nm in size; however, all nanoparticles were smaller than 300 nm, which may suggest that these particles may largely benefit from the so-called EPR effect when administered intravenously. Standard deviations of all formulations were very low, indicating unimodal monodisperse particle size distributions for both types of amphiphilic β-CD nanoparticles. However, β-CDC6 and PLGA nanoparticles appear to be more advantageous regarding their potential of sterilization by membrane filtration due to their small size and favorable polydispersity index value.

Zeta potential values are also of interest for the characterization of these nanoparticles since CPT-loaded nanoparticles display zeta potential values varying between 0 and −20 mV as seen in Table 3. Zeta potential is a function of the surface charge of colloidal dispersions. It is commonly used to predict and control dispersion stability. Zeta potential data suggest the potential physical stability of cyclodextrin nanoparticles in aqueous dispersion state and confirm the presence of CPT on particle surface. Imaging of nanoparticles by SEM is expected to provide information on nanoparticle morphology and size. Examination of SEM photographs of the nanoparticles revealed that the surfaces were smooth and spherical, as seen in Fig. 3.

Nanoparticles were prepared according to the nanoprecipitation method by high-loading technique based on preparing nanoparticles directly from preformed inclusion complexes [24]. Preparation of nanoparticles from preformed inclusion complexes of CPT and amphiphilic β-CD proved to be an effective method to enhance drug loading to nanoparticles. Data are expressed in terms of entrapped drug quantity, entrapment efficiency and associated drug percentage to clarify the amount of drug associated in the nanoparticles and the amount of drug entrapped per unit CD. Data suggest that 6-O-Capro-β-CD has a higher loading capacity than β-CDC6. Entrapment efficiency and associated drug percentage were significantly higher ($p < 0.05$) for 6-O-Capro-β-CD modified on the primary face. These results in Table 4 confirm that drug inclusion may be facilitated by leaving the secondary phase unmodified and thus reducing the steric hindrance and leaving the wider side of the cavity open for the entrance of CPT totally or partially, as suggested previously by Memisoglu et al. [24]. On the other hand, preparation of polymeric nanoparticles from preformed inclusion complexes of CPT and HP-β-CD resulted in lower drug-loading values as a result of the highly hydrophilic character of drug:cyclodextrin complex. Data suggest that PLGA nanoparticles have a higher loading capacity than PCL nanoparticles. This

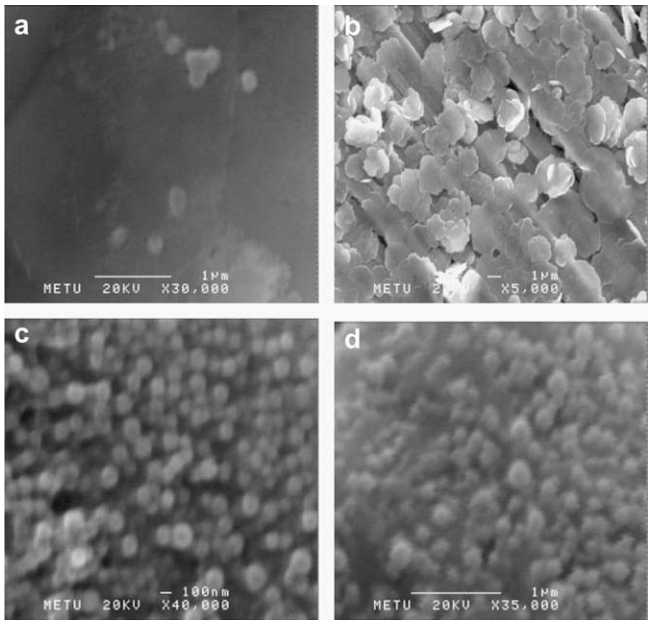

Fig. 3. SEM of nanoparticles (a: 6-O-Capro-β-CD, b: β-CDC6, c: PLGA, d: PCL nanoparticles).

Table 3
Mean diameter (nm), polydispersity index (PI) and zeta potential (mV) values of the cyclodextrin nanoparticles.

Formulations	Mean diameter (nm) ± S.D.	PI	Zeta potential (mV) ± S.D.
Blank β-CDC6 nanoparticles	130 ± 0.5	0.07	−16 ± 0.9
CPT:β-CDC6 nanoparticles	142 ± 3	0.28	−22 ± 0.7
Blank 6-O-Capro-β-CD nanoparticles	194 ± 4	0.2	−25 ± 1.3
CPT:6-O-Capro-β-CD nanoparticles	271 ± 15	0.41	−13 ± 0.9
Blank PLGA nanoparticles	178 ± 1	0.05	4,61 ± 2.76
CPT-loaded PLGA nanoparticles	187 ± 9	0.08	−0.06 ± 1
Blank PCL nanoparticles	189 ± 4	0.2	−12 ± 0.6
CPT-loaded PCL nanoparticles	274 ± 0.8	0.07	−19 ± 0.2

Table 4
Drug-loading values of the amphiphilic β-cyclodextrin nanoparticles loaded with CPT.

Formulations	Entrapped quantity (μg) ± S.D.	Associated drug% (± S.D.)
CPT:β-CDC6 nanoparticles	28 ± 2	9 ± 1
CPT:6-O-Capro-β-CD nanoparticles	48 ± 4	13 ± 3
PLGA nanoparticles	13 ± 0.1	2.6 ± 0.03
PCL nanoparticles	7.2 ± 0.5	1.4 ± 0.2

may be a result of the relatively more hydrophilic nature of PLGA when compared with that of PCL. It was found that CPT loading was 2.6% and 1.44% for PLGA and PCL nanoparticles, respectively. In general, it is seen that drug-loading values are significantly higher for amphiphilic cyclodextrin nanoparticles when compared with those of PLGA and PCL nanoparticles. Various nanoparticular drug delivery systems with CPT have been tried, and the resemble drug-loading properties were found. The entrapment efficiency of the nanoparticles reported were about 10–40% for CPT-loaded nanoparticles [14,36]. Kunii et al. reported that CPT-loaded nanoparticles were prepared using poly(DL-lactic acid) (PLA) and poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) copolymers (PEG–PPG–PEG), and the mean drug content was 1.6% (w/w) [36].

In vitro release profiles of CPT from different nanoparticles were assessed using the dialysis technique. It was demonstrated that the encapsulated drug was released from the nanoparticles in the lactone form and that the CPT was maintained in its stable lactone form for more than 12 days with cyclodextrin nanoparticles as seen in Fig. 4. Complete release of CPT was realized within a period of up to more than 144 h. On the other hand, 6-O-Capro-β-CD nanoparticles exhibited an initial release of approximately 30%, and the remaining CPT was liberated with a controlled release profile over 288 h, as seen in Fig. 4. The β-CDC6 nanoparticles released CPT faster and at a rate 2-fold higher than the 6-O-Capro-β-CD nanoparticles (Table 5). The more vigorous burst effect in the case of β-CDC6 nanoparticles resulted in 50% cumulative release of CPT after 5 h in contrast to 24 h in the case of 6-O-Capro-β-CD nanoparticles. The major significance of the CPT release profiles from both β-CDC6 and 6-O-Capro-β-CD nanoparticles is the fact that CPT was maintained in its active lactone form and released to the physiological medium for more than 12 days for 6-O-Capro-β-CD nanoparticles and 6 days for β-CDC6 nanoparticles. Nanoparticles consisting of preformed inclusion complexes are believed to release the drug entrapped in the CD cavity by time-dependent mechanisms such as dissociation upon dilution and competitive displacement of the drug from the cavity by release medium constituents, as well as by adsorption of the free drug from the nanoparticle surface and diffusion of the free drug from the

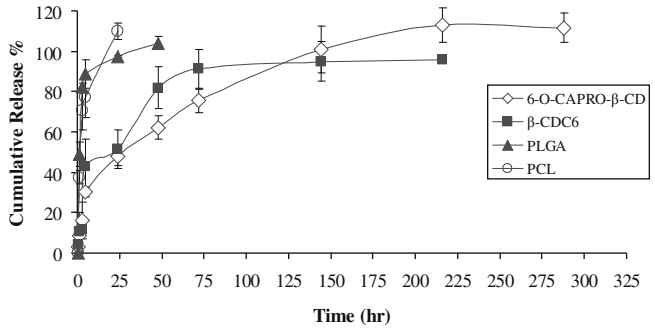

Fig. 4. In vitro release profile of CPT from nanoparticles into isotonic phosphate buffer, pH 7.4 (n = 3 ± S.D.).

Table 5
In vitro drug release time of nanoparticles.

	β-CDC6	6-O-Capro-β-CD	PLGA	PCL
30% release (burst effect)	2 h	5 h	0.5 h	1 h
50% release	5 h	24 h	1 h	2 h
Complete release	144 h	288 h	48 h	48 h

nanoparticles matrix structure [24]. It can be observed that the amphiphilic β-CD modification site has a significant effect on drug release. Amphiphilic β-CD modified on the primary phase, 6-O-Capro-β-CD, displayed slower release profiles than β-CDC6, modified on the secondary face. This difference is observed only for preloaded and highly loaded nanospheres. Both these techniques included preformed inclusion complexes; however, the slower release profile of 6-O-Capro-β-CD suggests that this molecule holds the drug in its cavity longer. In vitro CPT release profiles from polymeric nanoparticles are also shown in Fig. 4. It can be seen that PLGA nanoparticles liberate the drug in a considerably slower release profile than PCL nanoparticles. For PLGA nanoparticles, 50% of the drug was released within 1 h. Complete release of CPT lactone form was realized within a period of more than 48 h. For PCL nanoparticles, 70% of the drug was released within 3 h. CPT lactone form release was completed in 24 h. CPT–polymer interaction and hydrophilicity of the polymers are believed to play an important role in the release rate of CPT from PLGA and PCL nanoparticles. Cyclodextrin nanoparticles are believed to both entrap the drug in the cyclodextrin cavity and also as adsorbed on the particle surface. The release patterns were biphasic, comprising an initial first burst effect followed by a sustained, continuous phase. The first burst effect was attributed to the immediate dissolution and release of the portion of the drug located at or near the surface of the nanoparticles. In vitro release of CPT were delayed from cyclodextrin nanoparticles when compared with a previous study performed with chitosan and PLA/(PEG–PPG–PEG) nanoparticles in which release profiles were completed in 80 and 24 h, respectively [14,36,37]. CPT has a very low aqueous solubility (2 μg/mL); therefore, it is possible that the dissolution of CPT is the rate limiting step, resulting in the slow release rate observed. The drug was released up to 60 days with CPT-loaded nanohybrids [17]. A similar delayed-release profile was obtained when CPT was incorporated into our PEG-based hydrogel [15]. In vitro drug release from PLA/(PEG–PPG–PEG) nanoparticles was examined for 96 h using PBS. An initial rapid release of 19.6% followed by very slow release was observed [36].

The unpublished data of the hydrolysis kinetics studies showed that in PBS (pH 7.4, 37 °C), almost all of the parent drug (80%) was converted to the inactive carboxylate form after only 4 h. In contrast, after encapsulation of the drug to the nanoparticles, the entire drug fraction remained in its active lactone form in the nanoparticles during the in vitro release. The proportions of CPT in the lactone ring and carboxylate forms are critical in predicting tumor response to CPT because the lactone form has much higher antitumor efficacy compared with the carboxylate form. We therefore achieved the protection effect of CPT cyclodextrin nanoparticles on the CPT lactone ring form against hydrolysis under physiological condition (PBS, pH 7.4, 37 °C).

Anticancer efficacy of the nanoparticles on MCF-7 breast cancer cells is shown in Fig. 5. The cell viability at 1:128 dilution was 65% after treatment with CPT solution in DMSO, while it was found as 56% for CPT-loaded 6-O-Capro-β-CD, 61% for CPT-loaded β-CDC6, 81% for CPT-loaded PLGA, and 68% for CPT-loaded PCL nanoparticles. These results suggest that CPT is more effective when encapsulated into amphiphilic cyclodextrin nanoparticles, 6-O-Capro-β-CD in particular. Incubation time for the samples with MCF-7 cells was sufficient for drug release into the medium, and it was observed that

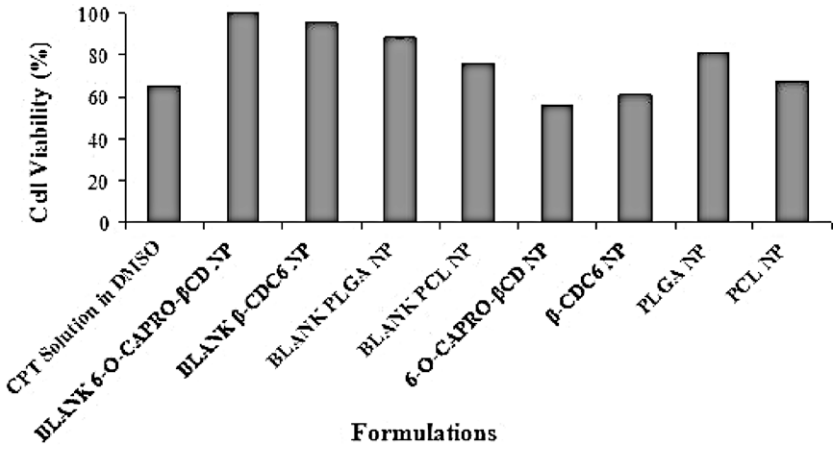

Fig. 5. Anticancer efficacy of cyclodextrin nanoparticles on MCF-7 cells (1/128 dilution). All assays were performed in triplicate, and mean OD values were used to estimate the cell viability. Cell viability (%) was calculated as (OD of treated wells/OD of untreated cells) × 100 (n = 3 ± S.D.) (CPT:Camptothecin, DMSO:Dimethyl sulfoxide, PLGA:Poly(lactide-co-glycolide), PCL:Poly-ε-caprolactone, NP:Nanoparticle).

cyclodextrin nanoparticles resulted in higher anticancer efficacy than PLGA and PCL nanoparticles. The cytotoxicity of blank nanoparticle formulations was evaluated in order to confirm that this difference did not arise from the cytotoxicity of the vehicle rather than the drug. The effects of unloaded β-CDC6, 6-O-Capro-β-CD, PLGA, and PCL nanoparticles on cell viability are seen in Fig. 6. The unloaded nanoparticles were found to be nontoxic on L929 cells at 1:128 dilution.

It is believed that formulating CPT with cyclodextrins could be advantageous to other polymeric material in the sense that formulation with cyclodextrins does not require the use of surfactants, which may add to the overall toxicity and complement the activation of nanoparticulate systems. Amphiphilic cyclodextrin nanoparticles resulted in more favorable in vitro characteristics including size, polydispersity, drug-loading, controlled release, stabilization of CPT and anticancer efficacy especially in the case of 6-O-Capro-β-CD modified on the primary phase. The degradation of PLGA causes a decrease in microclimate pH. The acidic microclimate of PLGA nanospheres has been identified to be the important source of stabilization of CPT. The pH is reported to strongly affect CPT stability [10].

4. Conclusion

In this study, a new nanoparticulate delivery system for CPT has been designed and compared with its polymeric analogues in terms of in vitro properties. CPT is among the most promising anticancer drugs developed in recent years. It has great potential as an anticancer agent provided the problems related to its physiological stability and insolubility can be solved. The results showed that cyclodextrin nanoparticles greatly increased the stability of CPT against hydrolysis and toward retention of the active lactone. Cyclodextrin nanoparticles were demonstrated to be a safe, effective, syringeable and membrane filterable due to their small size and stable formulation for delivery of CPT, and this approach represents a new platform for the delivery of other clinically important hydrophobic drugs. Thus, CPT-loaded cyclodextrin nanoparticles emerge as promising delivery systems for cancer therapy, and in vivo behavior of these nanoparticulate carriers for CPT should be further evaluated in animal models.

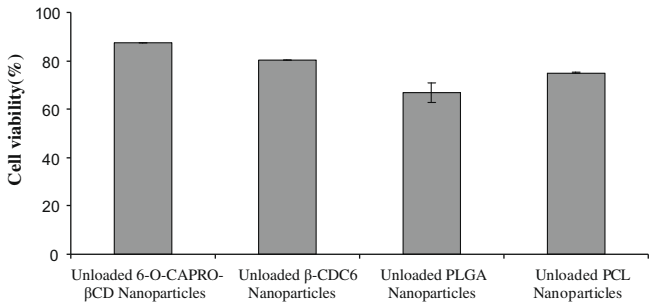

Fig. 6. The effect of unloaded nanoparticles on L929 cells (1/128 dilution) (n = 3 ± S.D.).

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