Cytotoxic effect of different camptothecin formulations on human colon carcinoma *in vitro*

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Two innovative 20-*S*-camptothecin (CPT) formulations, previously found suitable to achieve therapeutically relevant CPT concentrations, were assessed for their *in vitro* cytotoxic potential as compared to an aqueous CPT solution, using the MTT assay. The formulations, cationic CPT-containing liposomes (CPT-Lip), hydroxypropyl-β-cyclodextrin (HP-β-CD) complexed CPT (CPT-CD) and a saturated aqueous CPT solution (CPT-Sol), were diluted in culture medium to appropriate CPT concentrations (4.7–300 ng/ml), and incubated with HT-29 and SW-480 human colon carcinoma cell lines. IC_{50} values were calculated after 48 and 72 h incubation for the HT-29 and SW-480 cell lines, respectively, and were found to be of the same magnitude for all formulations, with only a slight difference (CPT-Sol < CPT-CD < CPT-lip). The cells obtained apoptotic morphology after 36 h incubation with CPT-CD and were demonstrated to be active caspase-3 immuno-positive. Both formulations investigated, CPT-CD and CPT-Lip, showed significant cytotoxicity *in vitro* relative to CPT-Sol and warrant investigation for future therapeutic application. *Anti-Cancer Drugs* **15:899–906**

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Introduction

Camptothecin (CPT) is a practically water-insoluble, naturally occurring alkaloid isolated from the leaves of *Camtotheca acuminata*, a tree that is native in China [1], and from *Mappia foetide* [2], a tree abundant in the Western Gaths of India. CPT was found to have potent antitumor activity in animal models [3], but clinical testing demonstrated only modest efficacy with significant toxicity. CPT itself as well as the first generation of CPT compounds (10-methoxy-CPT and 10-hydroxy-CPT) are extremely insoluble in water, but the sodium salt of CPT is water-soluble and was thus originally preferred for *in vivo* studies. However, it was later shown that this compound only possessed 10% of the cytotoxic activity of CPT-lactone form [4] and demonstrated several side-effects in phase I clinical trials. The interest in CPT therefore remained at a low ebb until 1985 when it was discovered that CPT, by a unique mechanism, inhibited the enzyme topoisomerase (Topo) I [5]. Topo I plays a crucial role in the normal replication of DNA by inducing a transient cut in one strand of DNA during the replication, through a reversible *trans*-esterification reaction, which yields a covalent intermediate form. This 'cleavable complex' normally lasts only long enough to allow passage of the newly synthesized strand through the formed cut after which the Topo I reseals the cleavage. Topo I inhibitors stimulate and stabilize this complex, causing strand scission and inhibition of the DNA replication [5–7].

A closed α-lactone ring within CPT (Fig. 1) is regarded an important structure prerequisite for both passive diffusion of the drug into cancer cells as well as for successful interaction with the Topo I target [8,9]. However, in human plasma CPT-lactone hydrolyses rapidly and almost completely to the ring-open carboxylate form [10,11]. The 100-fold higher binding affinity of CPT-carboxylate towards human serum albumin compared to the CPT-lactone causes a shift toward the ring open form in plasma [12], whereas lipid bilayers such as erythrocyte membranes are described to stabilize the lactonering and increase the $t_{1/2}$ in whole blood compared to plasma [13].

Many attempts have been undertaken to overcome the poor solubility and instability of CPT, the most successful being chemical modification and/or synthesis of prodrugs [5,14]. Furthermore, liposomes have been suggested as a suitable drug-delivery system for CPT, maintained to solubilize CPT and conserve the lactone ring of the molecule [15–20]. The second approach utilizing

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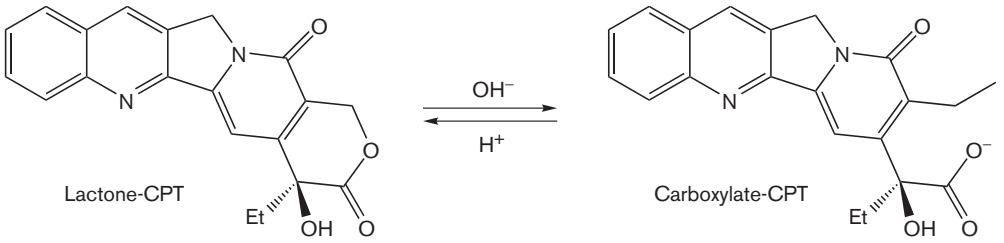
Fig. 1



The carboxylate–lactone equilibrium of CPT.

cyclodextrin (CD), e.g. hydroxypropyl (HP)-β-CD, forms a coin-like three-dimensional structure that provides a hydrophobic cavity, suitable to form inclusion complexes by weak non-covalent forces [21]. CD has a solubilizing effect and also stabilizes the lactone form of CPT, which is the more hydrophobic form of the drug [22]. To date, Irinotecan (Campto) and Topotecan (Hycamptin) are water-soluble, but still hydrolysable, CPT analogs that have reached the clinic with indication for advanced colorectal cancer, upper gastrointestinal malignancies and lung cancer (Irinotecan), and ovarian cancer and small cell lung cancer (Topotecan) [23]. Although CPT appears to have a good affinity both for liposomes and CDs, no such formulations have reached therapy so far. In the present study two novel formulations for CPT, a cationic liposome- and a CD-based one, both containing significant amounts of CPT, were evaluated for their *in vitro* cytotoxicity toward human colon carcinoma cells.

Material and methods

Reagents

S-(+)-Camptothecin (CPT) was obtained from Sigma-Aldrich (Munich, Germany). Egg phosphatidylcholine (EPC) was a kind gift of Lipoid (Ludwigshafen, Germany) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) was obtained from Avanti Polar Lipids (Alabaster, AL). HP-β-CD), Cavasol W7 HP Pharma, was obtained from Wacker-Chemie (München, Germany). MTT was purchased from Sigma (St Louis, MO). Dimethylsulfoxide (DMSO) was purchased from Sigma-Aldrich. Triethylamine (for analysis), acetic acid (glacial 100%), disodium hydrogen phosphate dihydrate (extra pure), potassium dihydrogen phosphate ('pro analysi') and sodium hydroxide pellets (extra pure) were purchased from Merck (Darmstadt, Germany). Triton X-100 was obtained from Sigma-Aldrich. Water was freshly distilled and buffers filtrated through 0.22-μm pore size filters before use. Organic solvents were obtained from Merck and had a gradient grade quality for liquid chromatography.

Cells

Human colon adenocarcinoma cells, HT-29 (ATCC, HTB-38) and SW-480 (ATCC, CCL.228), were cultured in monolayers in RPMI 1640 supplied with 10% fetal calf serum and antibiotics. The cultures were kept at 37°C in an atmosphere of 5% CO_2.

CTP-liposome (Lip) preparation

A detailed description of the preparation of the CPT-Lip is in progress to be reported elsewhere. In brief, 21.0 mg CPT was dissolved in about 5 ml DMSO and 12.0 g phospholipids, EPC and DOTAP (4:1 mol/mol) were dissolved in a chloroform:methanol blend (1:2, v/v). The solutions were mixed and spin frozen in liquid nitrogen, and placed in a pre-cooled freeze-dryer (Beta 2-16 equipped with a LMC-2 controller; Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). After freeze-drying, the vials were sealed and stored at −80°C. The freeze-dried PL-CPT cakes were brought to room temperature before adding 25 mM phosphate-buffered saline (PBS), pH 6.0, to a total sample weight of 35 g. The lipid dispersion was homogenized using a high-pressure homogenizer (APV Micron LAB 40; APV, Lübeck, Germany) at 70 MPa for 10 cycles [24]. The resulting vesicular phospholipids gel (VPG) formulations were autoclaved using a steam sterilizer (CertoClave Typ CV-EL 10L/12L; CertoClav Sterilizer, Traun, Austria). VPG was diluted 1:3 (w/w) with 25 mM PBS, pH 6.0. Liposome dispersions were ultracentrifuged at 100 000 *g* for 20 min at 25°C in an Optima LE-80 ultra-centrifuge; SW60 Ti rotor (Beckman, Palo Alto, CA) in order to remove CPT crystals. The supernatant containing CPT-Lip was used for *in vitro* studies after quantification of CPT by HPLC.

Control-Lip preparation

These liposomes were prepared by the same procedure as described for the CPT-Lip except that no CPT was added to the mixture. These liposomes were used as control in the cytotoxicity MTT study.

CPT-CD complex preparation

A detailed description of this formulation will be reported elsewhere. Briefly, CPT in excess and 25 mM phosphate buffer, pH 6.0, containing 20% (w/v) HP-β-CD, were sonicated for 30 min before incubation at 25 ± 0.5°C for 5 days at 70 r.p.m. in a shaking water bath, type GFL 1086 (GFL, Burwedel, Germany). The suspension was filtrated through a 0.22-μm Millex filter, (Millipore, Carrigtwohill, Ireland), the first drops discarded and the rest collected in sterile Sarstedt vials. The content of CPT in the CPT-CD sample was quantified by HPLC.

Phosphate-buffered CPT solution

This solution, CPT-Sol, was prepared by the same way as the CD complex, except that the solvent used was 25 mM isotonic PBS, pH 6.0.

HPLC method

A HPLC method, previously described by Warner and Burke [25], was used with some modifications. The mobile phase had a gradient from 25 to 35% acetonitrile during 10 min in a 1% triethylamine buffer, pH 5.5 and at a flow rate of 1 ml/min. The Waters HPLC system was equipped with a 474 scanning fluorescence detector, a 2695 separation module and a Symmetry C_{18} column, 3.9 × 150 mm (Waters, Milford, MA). Wavelengths: excitation $\lambda = 360$ nm and emission $\lambda = 440$ nm. Injection volume was 10 μl. A 3.33 mg/ml CPT stock solution for the standard curves was made in DMSO and the standard samples with CPT concentrations from 25 to 250 ng/ml made by dilution in 9 mM phosphate buffer, pH 10.5 and pH 3.0 for the carboxylate-CPT and the lactone-CPT, respectively. Every standard solution was prepared in triplicate and injected twice into the HPLC. Samples were diluted into the concentration range of the calibration curve with 25 mM PBS and acidified by 25 mM PBS, pH 3.0, for conversion of all CPT into the lactone form and quantification of the total CPT concentration in the samples. Liposome samples were added with 5% Triton (w/w) in PBS, pH 3.0, to dissolve the liposomes prior to analysis.

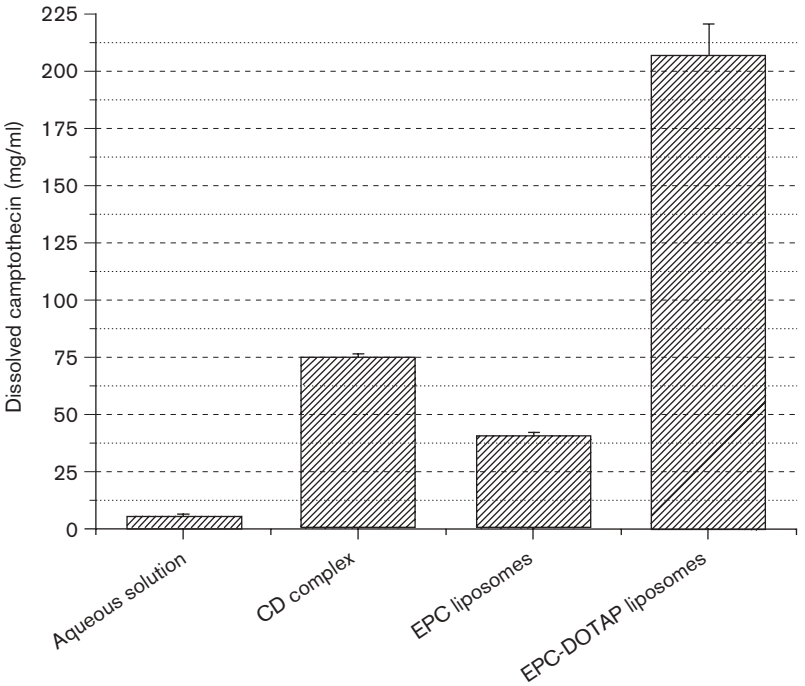
Cytotoxicity assay

Cytotoxicity of CPT formulations towards tumor cells, HT-29 and SW-480, was assayed by the MTT assay as previously described [26].

Immunocytochemistry

Identification of active caspase-3-positive cells was performed by using polyclonal anti-active-caspase-3 antibody (R&D Systems, Abingdon, UK). HT-29 and SW-480 cells were grown for 36 h on chamber slides (Nunc,

Fig. 2



The solubilization potential of three different CPT vehicles and aqueous solution at pH 6.0 prepared (25 mM phosphate buffer) at 25°C ($n = 3$ in solutions and $n = 4$ liposomes).

Roskilde, Denmark) without or in the presence of 150 ng/ml CPT-CD. The cultures were then washed and fixed for 12 h in 4% paraformaldehyde in phosphate buffer containing 0.2 M sucrose. After washing with TBS, the antibody was diluted 1:10 and incubated with the cell cultures overnight at 4°C. After rinsing in TBS, the cultures were incubated for 45 min with secondary biotinylated antibody and thereafter with FITC–streptavidin (Zymed, San Francisco, CA). The cultures were examined and photographed using a Zeiss Axiophot fluorescence photomicroscope (Carl Zeiss, Oberkochen, Germany) equipped with Nikon Coolpix 995 digital camera.

Result and discussion

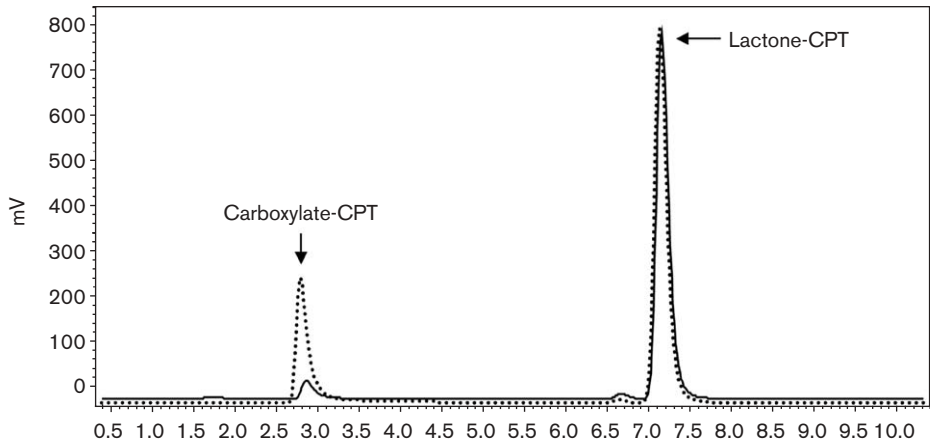
Use of CPT in a clinical setting has been hampered by modest efficacy and significant toxic side-effects. The principal problems include low solubility and low stability of the closed lactone form in human blood and tissue fluid, and binding to serum albumin. To improve the pharmacological profile of CPT, a number of chemical derivatives with better solubility and/or improved tissue stability have been synthesized [23,27–30]. Alternative approaches also include new drug formulations and carriers [18,31–36], resulting in changes of biodistribution and stability of the drug. Both strategies are expected to improve the therapeutic effect of CPT. In this study, two formulations containing the native drug CPT, which in prior studies were found to gain therapeutically relevant concentrations at 50 μg/ml or above (Fig. 2), were tested for their *in vitro* antineoplastic potential against two human colon carcinoma cell lines, HT-29 and SW-480. The liposome formulation CPT-Lip, having a lipid composition of 20:80 mol/mol DOTAP/EPC, was found to incorporate more than 5 times higher amounts of CPT compared to liposomes containing EPC (Fig. 2) and was also superior to all other liposome formulations investigated in terms of incorporation (results not shown). However, as DOTAP is a cationic lipid, the increased incorporation efficacy may theoretically be a result of ionic interactions between the cationic lipids and the anionic isomer of CPT. On the other hand, lipophilic bilayers of liposomes are previously described to stabilize the more hydrophobic lactone isomer [37]. As the antitumor activity of CPT is provided by the lactone form of the drug [9], this equilibrium is important for the cytotoxic potential of the formulations. The lactone–carboxylate equilibrium in liposomes (CPT-Lip) could not be measured by HPLC, as a rapid re-equilibration takes place during sample preparation and tensides (Triton was used) needed for HPLC quantification to dissolve the liposomes do preferentially stabilize the lactone form. The lactone–carboxylate equilibrium of the drug in a buffer (CPT-Sol) and CD in formulation (CPT-CD) was compared (Table 1). The stabilization effect of HP-β-CD on the lactone form is illustrated in Figure 3, showing an increased area under the curve (AUC) for the lactone top in the HPLC chromatogram

Table 1 Formulation characteristics for the formulations used in this study

Characteristic	Solution	CD complex	Liposome
CPT concentration (μg/ml)	5.2 ± 1.2	74.8 ± 1.5	206.7 ± 14.1
Relative lactone content (%)	84.7 ± 0.1	92.3 ± 0.2[a]	ND[b]
HP-β-CD content [mg/ml (M)]	–	200 (0.14)	–
Total lipid content (mg/ml)	–	–	85–100[c]

[a]With 25% w/v HP-β-CD.
[b]Due to rapid re-equilibrium upon dissolution of the liposomes by tensides or organic solvents, the relative concentration of the lactone form is not easily accessible experimentally by HPLC.
[c]CPT quantified after ultracentrifugation, removing CPT crystals and a minor population of the bigger liposomes (<15%).

Fig. 3



The HPLC chromatograms of CPT before (dotted line) and after (solid line) adding 25% (w/v) HP-β-CD.

when CD is present. HPLC analysis showed that at pH 6, CPT was 92% in the lactone form when 25% HP-β-CD (w/v) was present compared to 85% in the phosphate buffer.

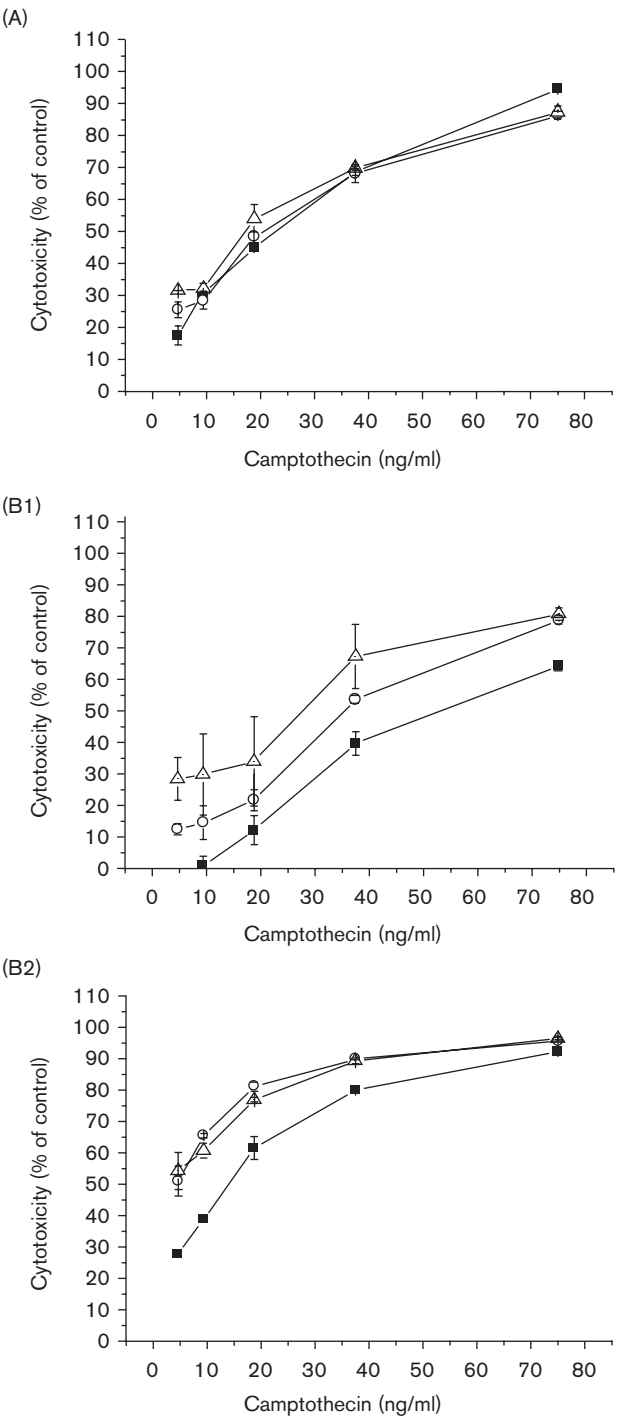
CPT of the CPT-Lip formulation was proven to be associated with the liposomes, as unassociated CPT crystals were removed by ultracentrifugation and the solubility of CPT in buffer only was below 3% as compared to the CPT concentrations reached in the liposome formulation (Table 1). The solubility of CPT in PBS is just high enough to reach cytotoxic concentrations in *in vitro* cell cultures, but for therapeutic concentrations *in vivo*, solubilization strategies such as HP-β-CD or liposomes are essential to reach therapeutically relevant concentrations.

The cytotoxic effect of CPT on the cell lines HT-29 and SW-480 cells grown in 96-well culture plates in presence of the three different CPT formulations added for 48 and 72 h, and assessed by the MTT assay is shown in Figure 4. A 24-h incubation did no lead to any significant cytotoxicity and thus was only performed initially, as longer incubation was needed. In preliminary studies for the MTT assay, the potential interaction of the medium of the formulations (HP-β-CD, liposomes and buffer) was investigated. Neither buffer nor CD controls gave any cytotoxicity in the concentration range applied, but the cationic liposomes showed some cytotoxicity after 72 h of incubation at the highest lipid concentrations that referred to CPT concentrations of 300 and 150 ng/ml (data not shown). The samples that contained 300 and 150 ng/ml of CPT were therefore excluded from the result sets, and only the lower concentrations used for cytotoxicity comparison (Fig. 4).

The MTT results that were obtained after 48 and 72 h incubation of the HT-29 and SW-480 cell lines, respectively, were found appropriate for calculating IC_{50} values, using the formulations and CPT concentrations applied in this study. The IC_{50} values are summarized in Table 2. A dose-dependent cytotoxicity was observed for all formulations, but the cytotoxic effect of CPT was consequently observed to be higher in the cell line HT-29, where the p53 proto-oncogene is mutated. This strongly indicates that the effect is at least partly independent of intact p53, which is in accordance with previous studies, where a p53-independent apoptosis and even selectivity of CPT towards tumors with p53 mutants has been reported [38,39].

Comparable *in vitro* studies that have been performed previously with other CPT-Lip formulations have generally used higher CPT concentrations and/or shorter incubation periods, and resulted in a lower degree of cytotoxicity. In the study of Sugarman *et al.* [31], more than 20% cell viability of Difi colon cancer and MDA-Panc3 pancreatic carcinoma cell lines still remained viable after 120 h of incubation, independent of CPT

Fig. 4



Cytotoxicity measured in the two cell lines, SW-480 (A) and HT-29 (B): triangles = CPT-CD, squares = CPT-Lip and circles = CPT-Sol. Result obtained after 48 (B1) and 72 h (A and B2).

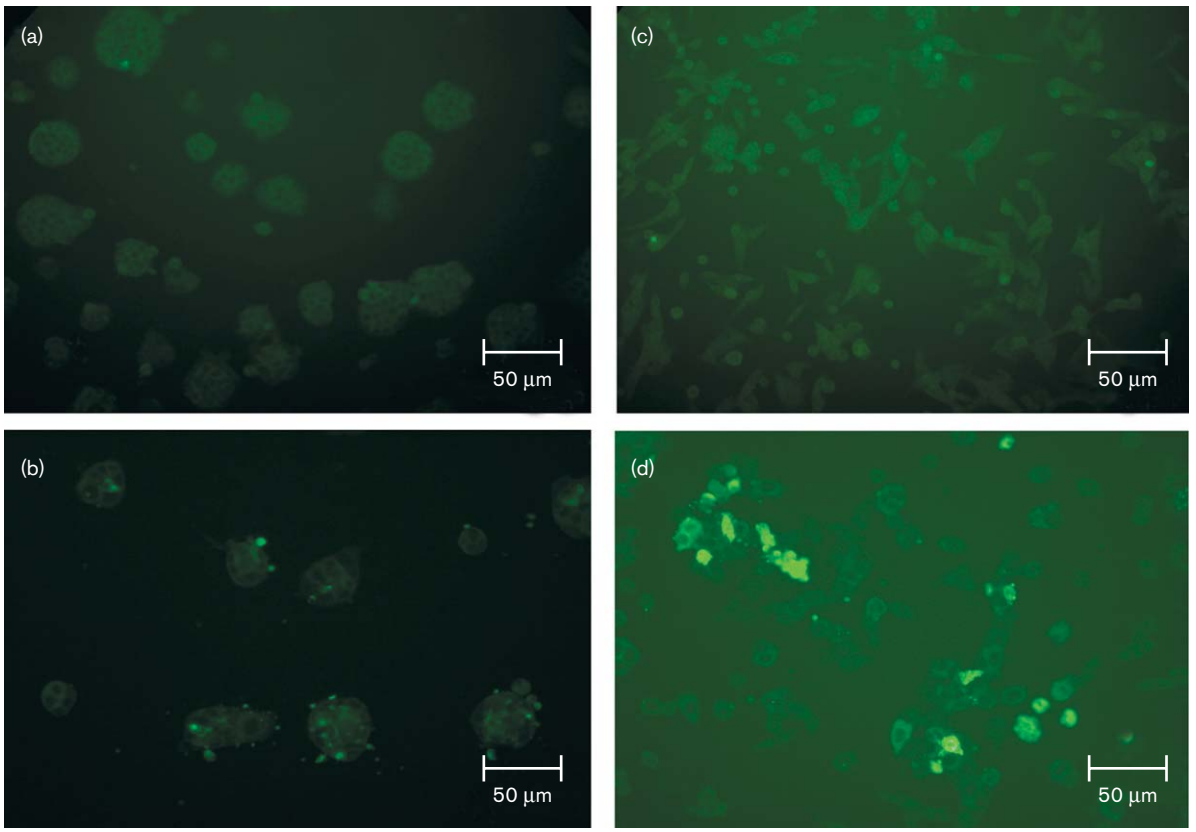
concentration, as an increase of CPT concentrations from 1 μM (348 ng/ml) did not significantly increase cytotoxicity. Cortesi *et al.* [16] incubated the cell cultures (human leukemic K562 cells) for 6 days, using comparable CPT concentrations of 0–100 nM (0–34.8 ng/ml), without reaching more than 80% cytotoxicity. Jones *et al.* [40] observed a slight decrease in cytotoxicity for CPT-containing liposomes *in vitro* in human breast carcinoma cell lines (MDA-MB-157, MDA-MB-231 and GI 101A), relative to free CPT. However, they concluded that this difference was basically time dependent and decreased when extending the incubation time beyond 24 h. Using a CPT concentration range of 1–2000 nM (0.35–696 ng/ml), they also observed a great variation in IC_{50} values between the different cell lines, both comparing IC_{50} values from results obtained by the MTT cytotoxicity assay and a [^{3}H]thymidine incorporation assay. *In vitro* cytotoxicity of CPT–HP-β-CD complex at concentrations from 1.0 nM to 10 μM (0.34 ng/ml to 3.48 μg/ml) has previously been described by Kang *et al.* [35], using the THP-1 cell line, assessing inhibition of [^{3}H]thymidine incorporation as a function of CPT concentrations after 20 h incubation. No cytotoxicity was observed with CPT concentrations lower than 100 nM (34.8 ng/ml), but at higher concentrations, the activity of the complex was increased compared to the free drug. In our study, a slight reduction of the cytotoxicity was generally observed for the CD complex as compared to CPT in buffer. However, in general, since different cell lines and growth media have been used in different studies, an absolute comparison is difficult to make. For the CD-complexed formulation, an increase in the *in vitro* cytotoxicity is unexpected, however, as a rapid dissociation of the complex is expected to take place after dilution in the growth medium [41].

Table 2 **IC_{50} values (ng/ml) of CPT formulations in the two cell lines, HT-29 and SW-480**

Cell line	HT-29 (48 h)	SW-480 (72 h)
CPT-Sol	28	17
CPT-CD	35	20
CPT-Lip	53	22

Caspase-3 is an effector caspase and mediates apoptosis by cleaving numerous structural and functional intracellular proteins such as lamin and caspase-activated

Fig. 5



Immunohistochemical detection of active-caspase-3 in HT-29 (b) and SW-480 cells (d) after treatment with CPT-CD (150 ng/ml CPT) for 36 h and untreated cells (a and c).

deoxyribonuclease inhibitor [42]. A marked immunoreactivity toward active-caspase-3 was observed in cultures incubated with CPT-CD (Fig. 5c and d) for 36 h, whereas control cultures were negative (Fig. 5a and b).

CPT-treated cells also displayed the characteristic morphological features of apoptosis, including chromatin condensation and membrane blebbing (Fig. 5). CPT-induced apoptosis seems to be partly dependent on caspase-3 and can occur via a p53-independent mechanism in HT-29 cells, as reported previously [43]. Recently, ceramide accumulation and subsequent apoptosis has been suggested to be involved in CPT-induced cytotoxicity in HT-29 cells [44]. In the present study, the formulation did not seem to affect the cytotoxicity of the drug, since both the cytotoxicity profile and IC_{50} values were comparable to the same extent. This is a promising aspect with further *in vivo* experimentation in mind, indicating that the lactone–carboxylate equilibrium of the drug in the preparations and uptake into the cells is not significantly altered by the formulations. Also, therapeutically relevant CPT concentrations (approximately 50 μg/ml) are achievable with both CD and liposomes, using physiological acceptable formulation characteristics (pH, isotonicity, particle size) (data not shown).

In conclusion, the two CPT formulations investigated, CD-CPT and CPT-Lip, are shown to have high cytotoxic activity against human colon carcinoma cell lines HT-29 and SW-480 upon incubation for 48 or 72 h. Both formulations show no major decrease in *in vitro* cytotoxicity compared to an aqueous solution of the drug and thus are judged promising for therapeutic application, where much higher concentrations will be needed. Such concentrations can be achieved by the liposomal or CD complex formulations, but not by the aqueous solution. Whereas liposomes hopefully will provide a mechanism to solubilize and stabilize CPT, and change its biodistribution in favor of a targeting effect towards solid tumors, the CD complex is not expected to change the pharmacokinetics of the drug significantly, as a dissociation of the drug from the CD cavity is expected to rapidly take place in plasma after i.v. administration.

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