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Formulation study for the antitumor drug camptothecin: liposomes, micellar solutions and a microemulsion

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Abstract

In the present paper we describe the production and characterization of specialized delivery systems for camptothecin, namely liposomes, micellar solutions and microemulsion. For instance, liposomes were prepared by reverse phase evaporation technique followed by extrusion through polycarbonate filters. Liposomes were characterized in term of dimensions, morphology and encapsulation efficacy. All the formulations were designed firstly to increase the solubility of camptothecin in aqueous environment and secondly to reduce the toxicity problems related to the administration of this drug. The analysis of their in vitro antiproliferative activity on cultured human leukemic K562 cells demonstrated that liposomes, micellar solutions and microemulsion containing camptothecin exert similar or slightly enhanced effect as compared to that shown by the free drug. Based on these results, the specialized delivery systems here proposed typify an interesting starting point for a future use in experimental therapy. © 1997 Elsevier Science B.V.

Keywords: Camptothecin; Liposome; Micelle; Microemulsion; Specialized delivery systems; Antiproliferative activity

1. Introduction

Camptothecin (CPT) is a potent cytotoxic alkaloid showing anticancer activity in several animal tumor models. This drug and its analogues (usually referred to as camptothecins), after failing to exhibit benefits in clinical studies with cancer patients in the early 1970s, are now re-emerging as promising drugs with multiple actions in the treatment of human malignancies. Camptothecins exert a broad antitumor activity both in preclinical as well as in phase I/II studies (Dancey and Eisenhauer, 1996). This class of compounds displays its activity by inhibiting the enzyme topoisomerase I forming a covalent enzyme–DNA

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complex, thereby resulting in a single-strand breaks (Bodley et al., 1995).

Camptothecins are usually administered either by continuous infusion or by multiple time-spaced injections, both resulting in a low patient comfort and compliance. In addition, the clinical use of camptothecins have some other practical disadvantages mainly due to (a) scarce water solubility and (b) a number of toxic effects. After prolonged administration camptothecins can indeed result in neutropenia, thrombocytopenia, anaemia and a number of non-haematological toxic effects such as alopecia, nausea, vomiting, diarrhoea, fatigue and skin rash (Dancey and Eisenhauer, 1996; Slichenmyer et al., 1993; Muggia et al., 1972).

Because of these drawbacks, the development of controlled delivery strategies could lead to significative advantages in the clinical use of these drugs.

With the aim of using innovative ways to administer CPT, possibly overcoming or alleviating the solubility, specificity and toxicity problems associated with its use, we compare here the use of liposomes, micellar solutions and a microemulsion as delivery systems. Liposomes, for instance, are known (a) to enhance drug cellular internalization, (b) to generally decrease unwanted systemic toxic effects and (c) to increase drug solubility in biological fluids, modulating at the same time the drug release profile. Furthermore, in the case of CPT, the utilization of phospholipid vesicles to carry the drug to target cells could have other advantages, such as specific release by passive or active targeting strategies (Gabizon, 1989). In addition to liposomes, micellar solutions and microemulsions have been also proposed as efficient strategies for drug delivery (De Oliveira and Chaimovich, 1993; Attwood, 1994). Micellar systems and microemulsions offer particularly several potential advantages as delivery systems, such as their solubilization capacity, stability and simplicity of preparation. These delivery approaches are in addition able to modulate both the pharmacokinetic and the bioavailability of the drug to result in an overall increase in the drug therapeutic index.

Moreover, the amphiphilic properties of the surfactants used for the preparation of both micellar solutions and microemulsion allow the solubilization of lipophilic substances, as well as a potential increase in the permeability of the drug through biological membranes, generally resulting in an augmented intracellular drug concentration (Kakemi et al., 1993). For instance, these factors could contribute to increase the gastrointestinal absorption of camptothecins allowing their oral administration.

Summarizing, in this paper we describe (a) the preparation and characterization of liposome-associated camptothecin, (b) the preparation of CPT containing micellar solutions or microemulsion and (c) a comparative analysis of in vitro performancies of these three specialized delivery systems. Throughout the text we use the term 'liposome-associated CPT' to describe liposomes carrying CPT instead of the term 'liposome-entrapped', as due to its lipophilicity, CPT is expected to be associated (intercalated) within the vesicle phospholipid bilayer.

2. Materials and methods

2.1. Chemicals

Camptothecin was from Sigma, USA. Egg phosphatidyl choline (Egg-PC) was purchased from Lipid Products (Surrey, UK). Cholesterol (CH) was from Sigma, USA. Positively and nega-

M.W.	Solubility	λmax	ε
348.4	CH_3Cl:CH_3OH 1:1 (w:w)	361	59

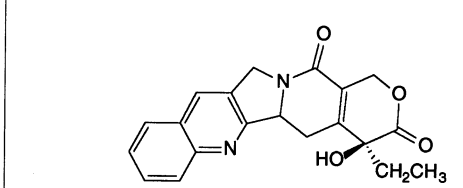

Fig. 1. Chemical structure and some physicochemical characteristics of CPT.

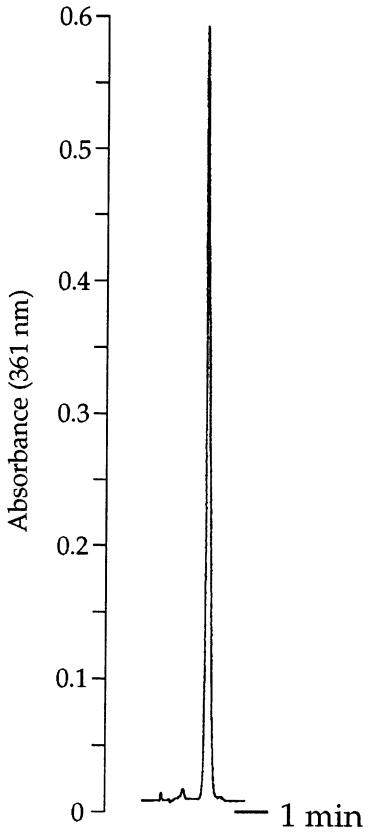

Fig. 2. Typical CPT chromatogram obtained from injection of the standard solution (100 μg/ml). Chromatographic conditions are described in the experimental section.

tively charged liposomes were produced using the cationic detergent didodecyl–dimethyl–ammonium bromide ($DDAB_{18}$) (Sigma, USA) and the anionic detergent dicetyl phosphate (DCP) (Lipid Products, Surrey, UK) respectively.

Poloxamer 407 (polyoxyethylene–polyoxypropylene block copolimer; 73:27 w/w) was supplied from BASF, Ludwigshafen, Germany.

Labrasol® (glycolysed ethoxylated C_8/C_{10} glycerides), Plurol isostearate® (polyglyceryl-6 diisostearate) and isostearyl-isostearate were supplied from Gattefossé, Milano, Italia.

All other material and solvent at the high purity grade were from Fluka (Buchs, Switzerland).

2.2. Preparation of liposomes

Liposome-associated camptothecin were prepared as follows. 100 mg of the mixture Egg-PC:CH 8:2 (mol/mol) or Egg-PC:CH:ionic surfactant 8:2:1 (mol/mol/mol) and 10 mg of CPT were dissolved in 1 ml of chloroform–methanol (2:1, v/v) in a 25 ml round bottom flask. After removal of solvent by rotary evaporation, the resulting dried lipid–CPT mixed film was dissolved in 4 ml of diethyl ether. To this solution, 2 ml of borate buffer pH 7.4 (5 mM $Na_2B_4O_7$, 180 mM H_3BO_3, 18 mM NaCl) were added and the mixture sonicated at 0°C (in an ice bath) for 10 min in a bath sonicator. The ether present was removed at room temperature by rotary evaporation under reduced pressure to give the final liposome aqueous dispersion.

The liposome dispersion was then injected into a device (Lipex Biomembranes, Vancouver, Canada) which allowed the extrusion of the vesicles through standard 25 mm diameter polycarbonate filters with 200 nm pore size (Nucleopore, Pleasanton, CA). The vesicles were extruded through two stacked filters employing nitrogen pressure of 10–20 bars, collected and re-injected three times.

In order to separate the free CPT from liposome-associated CPT, the liposome suspension was loaded on a gel filtration Sepharose 4B column (Pharmacia, Uppsala, Sweden) (1.5 cm diameter, 50 cm length) pre-equilibrated and eluted with borate buffer. The void volume peak fractions containing the liposome-associated CPT were collected and quantitated for liposome and CPT content. Each fraction from the Sepharose 4B column was checked for phospholipid content by TLC and for CPT content by HPLC analysis.

2.3. TLC analysis of phospholipids

TLC analysis of liposomal material was carried out on 10×10-cm HPTLC plates (Kieselgel 60 F_{254}, Merck). Chromatograms were developed in chloroform:methanol:water (65:25:4 v/v/v) and stained with iodine (phosphatidyl choline $R_f =$ 0.45). The phosphatidyl choline content was quantitated by comparison with standard solutions by photodensitometric scanning.

2.4. Morphology of liposomes

Liposome shape and surface characteristics were studied by freeze fracture electron microscopy and submicron particle size analysis using a Balzen BAF 300 at 10^{-5} Pa and a Malvern S4700 Version PCS (Malvern Instruments, England), respectively.

2.5. Preparation of micellar solutions

Micellar solutions were prepared as follows. Polyoxyethylene–polyoxypropylene block copolymer, 73:27 w/w (Poloxamer 407), polyoxyethylene sorbitanmonostearate (Tween 80), polyoxyethylene sorbitantrioleate (Tween 85) or a mixture of Tween 80:Tween 85 1:1 (w/w) were dissolved in water at the concentration of 5% (w/v). Consequently, an excess of CPT powder (300 μg/ml) was added to obtain a saturated micellar solution and magnetically stirred overnight at room temperature. Afterwards, the resulting saturated solution was filtered through a Durapore® 0.1 mm (Millipore) pore filter in order to remove the insoluble CPT. The final CPT concentration in micellar solutions was determined by HPLC analysis.

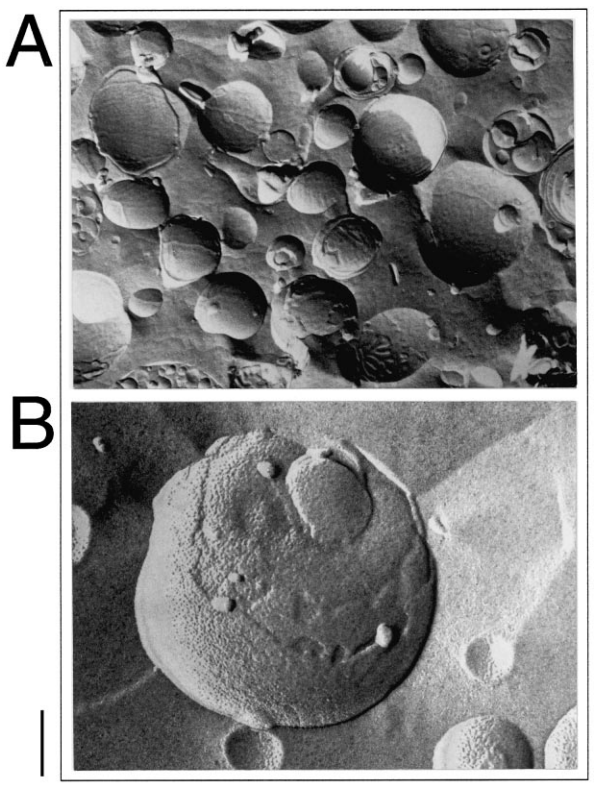

Fig. 3. Freeze-fracturing electron microphotographs of Egg-PC:CH liposome-associated CPT. Bar represents 200 nm (panel A) and 60 nm (panel B).

2.6. Preparation of the microemulsion

Microemulsions were prepared by mixing the following components: Labrasol® (glycolysed ethoxylated C_8/C_{10} glycerides; 33%, w/w), Plurol isostearate® (polyglyceryl–6 diisostearate; 13%, w/w), isostearyl–isostearate (12% w/w) and distilled water (42%, w/w). Since the formation of the microemulsion is a spontaneous event, only gentle stirring was required to obtain the formulation. Afterwards, 300 μg/ml of CPT powder were solubilized in the system resulting in a transparent stable liquid system.

2.7. HPLC analysis of CPT

The HPLC determination of CPT was performed using a Pharmacia gradient chromatographic pump, a Rheodyne 7125 sample injection valve (equipped with a 100-μl loop) and a Pharmacia VWM 2141 UV detector. Samples were chromatographed on a 100×4.6 mm reverse-phase stainless steel column packed with 5 μm particles (Model ODS Hypersil, Hewlett Packard, USA), eluted isocratically at room temperature with a mobile phase constituted for 65% of borate buffer pH 7.4 and 35% acetonitrile at a flow rate of 1 ml/min. CPT was monitored at 361 nm, the λ_{max} characteristic of the compound.

2.8. Cell growth studies

The effects of liposome-associated CPT and CPT containing micellar solutions and microemulsions were determined on in vitro cultured human leukaemic K562 cells (Lozzio and Lozzio, 1975). Standard conditions for cell growth were α-medium (Gibco, Grand Island, NY), 50 mg/l streptomycin, 300 mg/l penicillin, supplemented

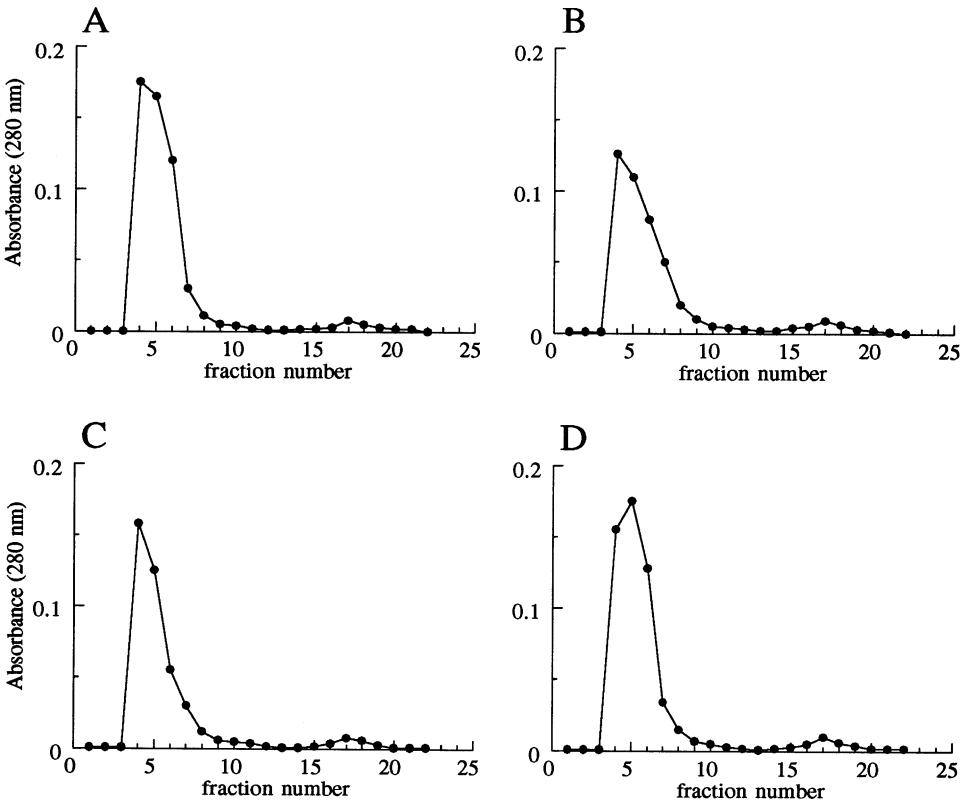

Fig. 4. Elution profiles of liposome-associated CPT from gel permeation chromatography on a Sepharose 4B column (1.3 cm internal diameter; 28 cm length; flow rate: 420 μl/min; 8.4 ml/fraction). The liposome-associated CPT were obtained by the two step method as described in the experimental section. A: Egg-PC liposomes; B: Egg-PC:CH liposomes; C: Egg-PC:CH:$DDAB_{18}$ liposomes; D: Egg-PC:CH:DPC liposomes.

with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA) in 5% CO_2 at 90% humidity. Cell growth was determined by counting with a Model ZF Coulter Counter (Coulter, Hielah, FL). Counts of viable cells were performed after 0.1% Trypan blue exclusion test.

3. Results and discussion

3.1. Determination of camptothecin by HPLC

Fig. 1 shows the chemical structure and some physicochemical characteristics of CPT. During the formulation study, CPT quantitation was performed by HPLC. To our knowledge no description has appeared in the literature for CPT determination by HPLC, but only for closely related compounds (Warner and Burke, 1997). Under the conditions described in the material and methods section, the retention time of CPT was 2.46 min. Fig. 2 shows the typical chromatographic profile of CPT.

Table 1
Yield of association of CPT to liposome

Liposome composition	Association yield (%)
Egg-PC	50
Egg-PC:CH[a]	57
Egg-PC:CH:$DDAB^{a}_{18}$	32
Egg-PC:CH:DCP[a]	38

[a] Liposomes molar ratio was Egg-PC:CH 8:2 (mol/mol) and Egg-PC:CH:ionic surfactant 8:2:1 (mol/mol/mol).

Table 2
Photon correlation spectroscopy analysis of liposome-associated CPT

Liposome composition treatment	Peak	Peak area (%)	Mean diameter (nm)	Width
Egg-PC				
Before extrusion	1	97.9	1996	112.0
	2	2.1	15 718	2353.3
After 200 nm pore filter extrusion	1	80.6	264	51.9
	2	19.4	628	89.5
After gel-filtration	1	86.8	242	30.1
	2	13.3	670	58.3
Egg-PC:CH (8:2 mol/mol)				
Before extrusion	1	35.5	2556	296.2
	2	64.5	8236	835.2
After 200 nm pore filter extrusion	1	96.9	195	1.9
	2	3.1	580	63.4
After gel-filtration	1	95.6	218	1.6
	2	4.4	484	33.3
Egg-PC:CH:$DDAB_{18}$ (8:2:1 mol/mol/mol)				
Before extrusion	1	100.0	252	3.2
After 200 nm pore filter extrusion	1	86.6	226	25.4
	2	13.4	658	60.8
After gel-filtration	1	96.7	216	1.7
	2	3.3	478	33.5
Egg-PC:CH:DCP (8:2:1 mol/mol/mol)				
Before extrusion	1	70.0	284	3.4
	2	29.6	584	58.7
After 200 nm pore filter extrusion	1	97.0	156	10.9
	2	3.0	348	73.5
After gel-filtration	1	96.6	212	39.6
	2	3.4	626	75.5

3.2. Liposomes

The preparation of liposome-associated CPT was conducted by a two step method based on a reverse phase evaporation (Szoka and Papahadjopoulos, 1978) followed by extrusion of liposomes through polycarbonate filters (Mayer et al., 1986). Liposome extrusion was performed using membranes with 200 nm pores. Liposomes were produced using different lipid compositions, namely Egg-PC or Egg-PC:CH (neutral liposomes), Egg-PC:CH:$DDAB_{18}$ (cationic liposomes) and Egg-PC:CH:DCP (anionic liposomes). A typical example of liposomes produced by this approach is reported in Fig. 3. Freeze-fracture electron microscopic analysis demonstrates that after extrusion, the liposomal suspension was constituted mainly of unilamellar vesicles with an average diameter reflecting the pore size of the membrane utilized for the extrusion.

In order to obtain information on the possible absorption of liposomal material to polycarbonate filters during the extrusion step, we performed a TLC analyses of liposome samples before and after extrusion. These experiments demonstrated that the liposome yield was almost quantitative in

Table 3
Aqueous solubility of CPT in micellar solutions

Solubilizing detergent	C.M.C. (g/l) (20°C)	H.L.B.	Solubilized CPT (μg/ml)	Increase of solubility[a]
Poloxamer 407 (5%, w/v)	0.5	—	2.6	2
Tween 80 (5%, w/v)	0.014	15	4.9	3.8
Tween 85 (5%, w/v)	0.025	11	4.8	3.7
Tween 80:tween 85 (5%, w/v)[b]	—	13	6.6	5

[a] The value represents the increase in solubility compared to water (1.3 μg/ml).
[b] The Tween 80:tween 85 mixture was 1:1 w/w.

term of phospholipid concentrations, but a 5% decrease in the final volume after each extrusion cycles through polycarbonate filters was observed.

Fig. 4 reports the elution profiles of the gel-permeation chromatography performed in order to separate the liposome-associated drug from the free CPT. As clearly appreciable, two major peaks with different relative heights are present in all the chromatograms. The first large peak, associated with the void volume of the column, reflects the elution of liposome-associated CPT. The identical elution volume obtained for all the liposome preparations suggests that the liposome size was similar for all the lipid composition used. The second, much smaller peak reflects the elution of the free drug. The presence of liposomes in the first peak was indicated by the turbidity of the solution and proven by TLC analysis (detecting the presence of phospholipids) and by electron microscopy. The presence of CPT in both peaks was proven by HPLC analysis of the corresponding fractions. From TLC experiments we could conclude that the second peak was free of phospholipids. The percentage of association of CPT to liposomes (encapsulation yield) was found rather high for all the tested lipid composition ranging from 32% in the case of positively charged liposomes to 57% in the case of neutral liposomes (see Table 1). The differences in the degree of association could be reasonably explained considering the charge of the liposomes. It appeared clear that a neutral lipid composition assured a higher efficiency for the camptothecin incorporation in liposomes.

Table 2 records liposome sizes after various treatment such as extrusion and gel-filtration.

3.3. Micellar solutions

It is well known that micellar solutions influence the solubility and the stability in water of lipophilic compounds. Lipophilic drugs can indeed be solubilized by the hydrophobic environment inside the micelles. The choice of the detergents used to produce the micellar solutions have been made on the basis of screening of several molecules. Detergents able to improve the solubilization of CPT in water were selected. Low molecular weight surfactants such as polyoxyethylene sorbitanmonostearate (Tween 80) and polyoxyethylene sorbitantrioleate (Tween 85) and a mixture 80:85 1:1 (w/w) were employed. In addition, the polymeric high molecular weight surfactant Poloxamer 407 was used. This latest compound is a block copolymer characterized by a molecular weight of 12 600 with a central polyoxypropylene hydrophobic portion surrounded by two hydrophilic blocks constituted of polyoxyethylene, where the proportion of polyoxyethylene by weight is about 73% (Garcia Sagrado et al., 1994). Solubility experiments were carried out by adding an excess of drug to 5% surfactant solutions. From the analysis of the results reported in Table 3, the mixture 80:85 1:1 (w/w) appears to give the better result in term of CPT solubilization. This formulation gave a fivefold increase of solubility as compared to CPT solubility in water.

3.4. Microemulsion

As reported in Table 3, the use of micellar solution resulted in a relatively low increase of

CPT aqueous solubility. CPT, in fact represents a challenging molecule being scarcely soluble both in aqueous solution and in organic solvents. Microemulsions could offer a more favourable environment for CPT solubilization. Microemulsions are indeed biscontinous systems essentially composed of bulk phases of water and oil separated by a surfactant rich interfacial region. The prepared microemulsion was a thermodynamically stable, single-phase, transparent liquid system composed of a surfactant (Labrasol®), a co-surfactant (Plurol isostearate®), an oil (isostearyl–isostearate) and water. After addition of 300 μg/ml of CPT a clear stable liquid system was obtained. 300 μg/ml does not represent the saturation limit for the microemulsion, in fact concentrations up to 500 μg/ml of the drug can be reached without any sign of precipitation. The microemulsion stability was evaluated for 30 days at room temperature. After this period, neither changes in colour, phase separation, creaming or CPT precipitation were detectable.

In this respect, the microemulsion was found an optimal formulation for CPT. CPT solubility in microemulsion was, in fact, at least five-fold higher with respect to that displayed by the micellar solution containing the mixture of polysorbate 80:85 1:1 (w/w) and almost 23-fold higher than that in water.

3.5. In vitro activity of CPT containing formulations

In vitro antiproliferative activity of CPT in liposomes, micellar solutions or microemulsion based formulations was determined and compared with that of the free drug. Human erythroleukaemic K562 cells were treated with the

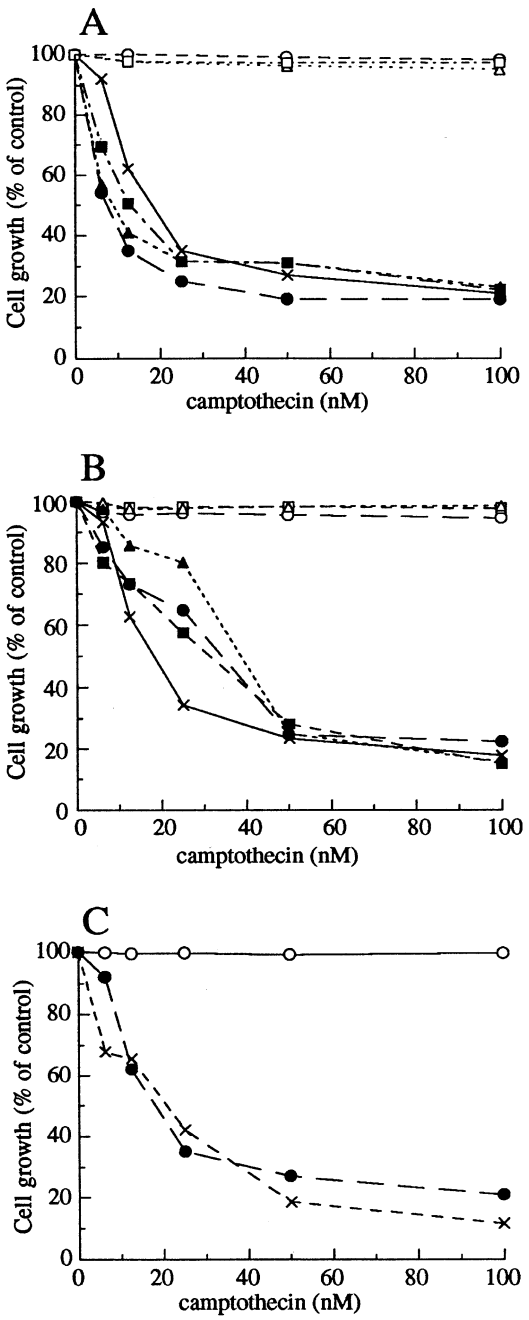

Fig. 5.

Fig. 5. Comparative analysis of the effect of free CPT (X) and CPT formulated in specialized delivery systems on proliferation of K562 cells. The empty and CPT containing formulations were added at the same indicated concentrations. Determinations were performed after 6 days of cell culture. Data represent the average of three independent experiments. A. Liposome: CPT/Egg-PC (●), empty Egg-PC (○); CPT/Egg-PC:CH:DDAB$_{18}$ (▲), empty Egg-PC:CH:DDAB$_{18}$ (△); CPT/Egg-PC:CH:DPC (■), empty Egg-PC:CH:DPC (□). B. Micellar solutions: CPT/Poloxamer 407 (●), empty Poloxamer 407 (○); CPT/Tween 80 (■), empty Tween 80 (□); CPT/Tween 80:Tween 85 (1:1 w/w) (▲) and empty Tween 80:Tween 85 (1:1 w/w) (△). C: Microemulsion containing CPT (●) and empty microemulsion (○).

same concentrations, in term of CPT molarity, of free or formulated CPT. After 6 days of cell culture, cells were electronically counted. Fig. 5 reports the results of these experiments, showing that CPT delivered by liposomes (panel A), micellar solutions (panel B) and a microemulsion (panel C) showed an antiproliferative activity similar or slightly enhanced with respect of that exerted by the free drug.

As control, the possible antiproliferative effects of the empty delivery systems on K562 cells was evaluated. The obtained data demonstrated that all the vehicles do not cause any inhibition of cell growth.

3.6. Concluding remarks

Recently, a large number of studies have focused on production of drug delivery systems for antitumor drugs. This effort was mainly done in reason of the scarce selectivity and high toxicity that characterize many (if not all) the antitumor drugs at the moment in clinical use, resulting in a limitation of their dosage and effectiveness. In this respect, the preparation and characterization of specialized delivery systems, such as liposomes, micellar solutions and microemulsion here proposed could represent a promising starting point for future utilizations in experimental therapy.

Acknowledgements

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