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The solubilization of lipophilic derivatives of podophyllotoxins in sub-micron sized lipid emulsions and their cytotoxic activity against cancer cells in culture

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Abstract

Sub-micron sized lipid emulsions with lipophilic drugs entrapped in the oil core are a novel drug carrier system with many potential applications. In this study, lipophilic derivatives of the anticancer drugs etoposide and teniposide, with an unsaturated fatty acid esterified to the phenolic 4′-hydroxyl, were solubilized in lipid emulsions. The emulsion droplets formed (particle diameter 104 nm) showed good physical stability and no leakage of entrapped drug. The fatty acid derivatives were chemically stable in the lipid emulsion preparations in both buffer and serum-containing medium. The cytotoxic activity of the drug emulsions was assessed in vitro against K562 and T-47D cancer cells and compared to the effect of the parent drugs under the same conditions. The linoleic acid derivative of etoposide showed very much the same cytotoxic activity as the parent drug while the oleic acid derivative of teniposide demonstrated a very low activity against K562 cells and only modest activity against T-47D cells. These fatty acid derivatives of the highly potent anticancer agents etoposide and teniposide represent a valuable addition to the supply of drugs suitable for incorporation into drug delivery emulsions.

Key words: Drug delivery; Cancer drug; Emulsion; Podophyllotoxin; Prodrug; Lipophilic derivative

1. Introduction

The lack of drug selectivity is a major problem in systemic cancer chemotherapy and targeting of cytotoxic agents to neoplastic cells would produce dramatic therapeutic gains. However, selective drug delivery of anticancer agents poses substantial technical difficulties (see Poznansky and Juliano, 1984). Particulate drug carriers such as liposomes and nanoparticles have long been extensively investigated as delivery systems for parenteral drug therapy (O'Mullane et al., 1987). Recently, oil-in-water (o/w) emulsions have aroused interest as a potential carrier system (Davis et al., 1987). Future applications could include sustained release, solubilization of lipophilic drugs and, above all, passive (or natural) targeting to macrophages (Singh and Revin, 1986) as well as active targeting by ligands attached to the surface of the particle (Iwamoto et al., 1991).

A previous study from this laboratory described the preparation of stable sub-micron sized lipid emulsions with an oil core of neutral lipid

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stabilized by phospholipid which was suitable for parenteral administration (Lundberg, 1994). Such o/w emulsions can solubilize a considerable amount of lipophilic drug in the hydrophobic oil core. Etoposide (VP-16) and teniposide (VM-26) are podophyllotoxin derivatives with low water solubility which necessitates the use of various solubilizers in their pharmaceutical formulation (Canetta et al., 1982). However, in spite of the poor water solubility of etoposide it was not possible to solubilize the drug in a lipid emulsion with a cholesteryl ester as oil component (Halbert et al., 1984). In the present work, lipophilic fatty acid derivatives of etoposide and teniposide were investigated. The drugs were solubilized in lipid emulsion droplets and the resulting preparations were assessed for physical and chemical stability as well as for cellular uptake and cytotoxic activity against cultured cancer cells.

2. Materials and methods

2.1. Materials

High-purity (99%) egg phosphatidylcholine (EPC), triolein, oleic acid, and cholesteryl oleate were purchased from Sigma Chemical Co. (St. Louis, MO) and polysorbate 80 was obtained from Fluka Chemie AG (Buchs, Switzerland). The radiolabelled compounds glycerol tri[9,10(n)-[^{3}H]oleate, [1-^{14}C]oleic acid, and cholesteryl [^{14}C]oleate were supplied by Amersham International plc (Amersham, U.K.). The cytotoxic compounds etoposide (VP-16), 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-ethylidene-β-D-glucopyranoside), teniposide (VM-26), 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidine-β-D-glucopyranoside), etoposide 4′-linoleate and teniposide 4′-oleate were provided by Bristol-Myers Squibb Co. (Wallingford, CT). Teniposide 4′-[1-^{14}C]oleate was synthesized in this laboratory from [^{14}C]oleic acid and teniposide. [^{14}C]Oleic acid chloride, prepared by use of oxalyl chloride, was quickly added to a solution of teniposide and *N,N*-diisopropylethylamine in dry acetonitrile. The mixture was stirred at room temperature for 45 min and then partitioned with phosphate buffer and ethyl acetate. After evaporation of the ethyl acetate, the resulting white powder was purified by preparative silica gel TLC. The structure of the drugs was characterized by NMR and MS and their purity were confirmed by TLC and HPLC. All tissue culture media were from Gibco Biocult (Paisley, U.K.).

2.2. Preparation of drug emulsions

The two lipophilic derivatives, etoposide 4′-linoleate and teniposide 4′-oleate, were incorporated into lipid emulsions according to a method described in detail elsewhere (Lundberg, 1994). Briefly, the emulsion components, 0.4 mg drug (etoposide 4′-linoleate or teniposide 4′-oleate), 1.6 mg triolein, 1 mg EPC, and 0.4 mg polysorbate 80, were dispersed into vials from stock solutions stored at 70°C, the solvent was evaporated and the samples vacuum-desiccated overnight. Subsequently, 2 ml of phosphate-buffered saline, PBS (NaCl, 0.14 M; KCl, 2.68 mM; $CaCl_2$, 0.9 mM; $MgCl_2$, 0.5 mM; Na_2HPO_4, 8.1 mM, and KH_2PO_4, 1.5 mM; pH 7.4) was added to the samples and the mixture was then sonicated (3 × 20 s) at 30°C using an MSE sonifier equipped with a titanium probe.

2.3. Characterization of emulsion particles

The emulsion droplet size was measured by quasielastic laser light scattering using a Malvern 4700 submicron particle analyzer. Prior to the measurements, the samples were filtered through a 0.22 μm filter to remove impurities. The loss of drug during this procedure was less than 5%. The integrity of the drug emulsion droplets was checked by passing samples containing teniposide 4′-[^{14}C]oleate and alternatively [^{3}H]triolein through a Sephacryl 300 column (30 × 2 cm) eluted with PBS. The elution profiles of the emulsion preparations were recorded by quantitation of the radioactivity in the fractions by scintillation counting in a 1216 Rackbeta scintillation counter (LKB-Wallac, Turku, Finland) with OptiPhase 'HiSafeII (LKB Scintillation Products) as scintillation cocktail.

The colloidal stability of the emulsions was determined by measurement of the particle size after storage of sterile samples at 4°C for different times. The chemical stability of the ester bond between teniposide and [^{14}C]oleic acid in the lipid emulsions was evaluated by quantitative TLC.

2.4. Cell culture

T-47D breast cancer cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acid solution, 0.08% (w/w) sodium bicarbonate, streptomycin (10 μg/ml), penicillin (10 μg/ml), and 10% (v/v) fetal calf serum (FCS). K562 erythroleukemic cells were maintained in RPMI 1640 medium supplemented with 5% (v/v) FCS, streptomycin (10 μg/ml), penicillin (10 μg/ml) and 2 mM L-glutamine. Cells were maintained at 37°C and gassed with 5% (v/v) CO_2 in air. The experiments were carried out with cells in the exponential growth phase.

2.5. Cellular uptake and hydrolysis of drug

The uptake of drug from emulsion globules by K562 and T-47D cells in culture was studied by incubation of the cells with preparations containing teniposide 4′-[^{14}C]oleate at 37°C for 4 h. The experiments were performed in 35-mm Petri dishes with serum-free growth medium. Emulsions containing cholesteryl [^{14}C]oleate were used as comparison in order to clarify the relative contribution of cellular uptake by whole droplets respective surface transfer. Cholesteryl esters are non-exchangeable compounds and in a serum-free medium their cellular uptake from emulsions will depend solely on the incorporation of whole globules. Emulsions containing teniposide 4′-[^{14}C]-oleate were also incubated with cells in growth medium containing 10% FCS. After the completion of incubation, the T-47D cells were washed three times with cold PBS and then detached by trypsin treatment. The cells were collected on Whatman GF/C filters and washed twice with PBS. The filters were dried for 30 min in an oven at 50°C, transferred to scintillation vials, and counted for radioactivity. The K562 cells were harvested by low-speed centrifugation and washed three times with cold PBS before scintillation counting.

The cellular hydrolysis of teniposide 4′-[^{14}C]oleate was measured by quantitative TLC. The cells were incubated for 4 h with emulsions at a drug concentration of 10 μM. After incubation had been completed, the cells were harvested, lyophilized and thoroughly extracted with chloroform/methanol (2:1). The intact drug and liberated fatty acid were separated by quantitative TLC and detected with I_2 vapour. The spots were cut into scintillation vials and counted for radioactivity.

2.6. Cytotoxicity testing

The ability of the drugs to inhibit cell proliferation was measured by determination of [^{3}H]thymidine incorporation. The parent drugs, etoposide and teniposide, were added to the incubation medium dissolved in ethanol/DMSO (1:1, v/v). The fatty acid derivatives of the drugs were added in the form of drug emulsions. Control cultures were treated with drug-free emulsions and solvents, respectively. At 3 h prior to the end of the experiment, 1 μCi [^{3}H]thymidine/ml was added to the incubation medium. After the completion of incubation, the suspended cells were collected on Whatman GF/C filters and washed with cold PBS. The filters were dried for 30 min in an oven at 50°C and the dried filters were transferred to scintillation vials and counted for radioactivity. Cells harvested in this way yield similar but quantitatively greater values than by using sonication and subsequent precipitation of the acid-insoluble macromolecules with 10% trichloroacetic acid (Leung et al., 1981).

2.7. Analytical procedures

The concentration of unlabelled drug was evaluated by HPLC using a 25 cm UltraTechsphere 5-ODS column (HPLC Technology Ltd) eluted with acetonitrile/water (60:40) at a flow rate of

1 ml/min. The liquid chromatograph consisted of a Spectroflow 400 solvent delivery system and a Spectroflow 757 absorbance detector (Kratos Analytical Instruments) (set at 254 nm) coupled to a Shimadzu C-R3A Chromatopac integrator. The concentration of teniposide 4′-[^{14}C]oleate was calculated from the radioactivity using the specific activity of the labelled compound. The octanol/water partition coefficient was determined by the shaking-flask method. Preparative TLC was performed on silica gel G plates developed with $CH_2Cl_2/CH_3OH/HAc$ (90:9:1) and quantitative TLC on Kieselgel 60 (DC-Alufolien, Merck) with the same developing solvent. Protein was assayed according to a modified Lowry method (Markwell et al., 1978) using albumin as standard.

3. Results and discussion

3.1. Drugs

Drugs intended for a stable incorporation into the oil core of lipid emulsions must be highly lipophilic. Etoposide, with a water solubility of about 0.25 mg/ml at 20°C (Canetta et al., 1982) and an o/w partition coefficient ($K_{o/w}$) of 6, does not meet these criteria and will leak out from lipid emulsions (Halbert et al., 1984). The same behaviour can also be expected for teniposide, although this drug has a somewhat higher $K_{o/w}$ of 44. On the other hand, the fatty acid derivatives of these drugs proved to be sufficiently hydrophobic for successful solubilization in lipid emulsion droplets. In these derivatives an unsaturated fatty acid is esterified to the phenolic hydroxyl in the C-4′ position of the epipodophyllotoxin glucopyranosides (Fig. 1). The esterification with oleic acid increased the $K_{o/w}$ of teniposide to 1780, and produced an oil-soluble compound. However, initial tests showed that emulsions prepared with the fatty acid derivatives as the sole oil component resulted in coarse and unstable emulsions. This problem was solved through a series of experiments which clarified that solubilization of the drugs in a 4-fold weight excess of triolein yielded proper emulsions.

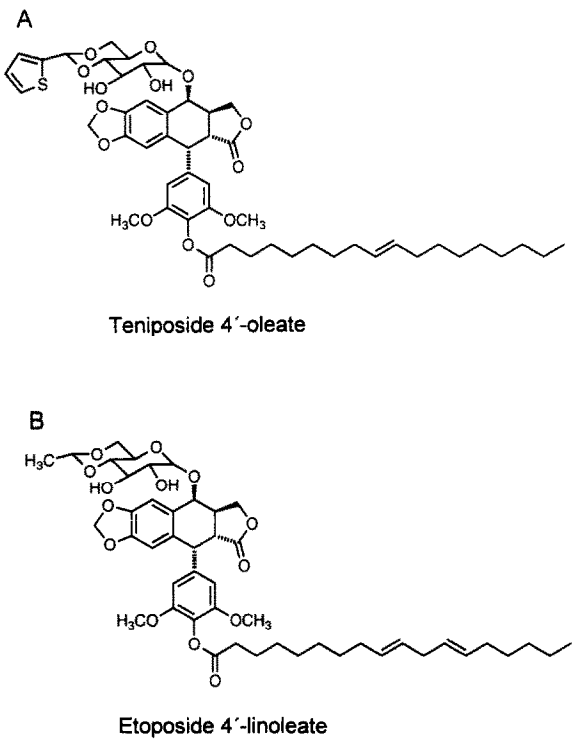

Fig. 1. Chemical structure of the fatty acid derivatives of etoposide and teniposide; (A) teniposide 4′-oleate and (B) etoposide 4′-linoleate.

3.2. Physical and chemical stability of drug emulsions

The size of emulsion particles intended for use as drug carriers is of critical importance. Colloidal stability as well as cellular uptake and in vivo distribution are highly dependent on this property (Poste et al., 1982). The desired particle size of about 100 nm was successfully achieved and 80% (by number) of the emulsion globules were in the size classes between 60 and 100 nm, with a mean diameter of 104 ± 3 nm (mean ± SD, $n = 5$, distribution of mass). Droplets containing etoposide 4′-linoleate or teniposide 4′-oleate were in the same dimensional range. The emulsion preparations were fairly monodisperse and the polydispersity (P), calculated from the equation, $P = r/r_N$, where r_N is the number mean and r denotes the mass mean, gave a value of 1.20.

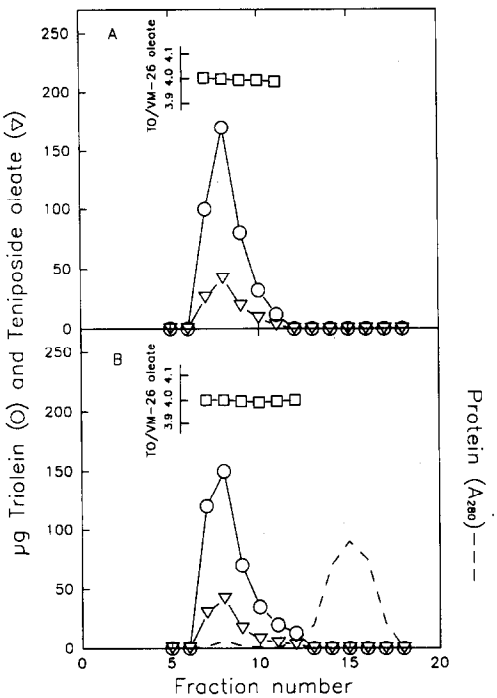

Fig. 2. Characterization of a representative drug emulsion preparation by Sephacryl 300 column chromatography. (A) Elution profile of a preparation containing [^{3}H]triolein (○) and teniposide 4′-[^{14}C]oleate (▽) as core components. (B) A preparation identical with (A) but incubated with serum for 1 h at room temperature (broken line represents protein recorded as A_{280}).

The physical stability of the drug emulsions and the possible leakage of drug out of the emulsion droplets were examined by gel chromatography on a Sephacryl 300 column. The data presented in Fig. 2A demonstrate that the particles eluted as a single symmetrical peak with complete coelution of the core components [^{3}H]triolein and teniposide 4′-[^{14}C]oleate and with a weight ratio intermediate between those of the two components identical with that of the applied preparation. This finding must be regarded as strong evidence for the integrity of the emulsion droplets and rules out leakage of drug from the emulsion particles. The same behaviour was also noted after incubation of the preparation in serum for 1 h (Fig. 2B). Furthermore, no measurable radioactivity was noted in the major protein peak, eluting after the emulsion particles, excluding protein binding of the drug. It can thus be concluded that the fatty acid derivatives, in contrast to free etoposide (Halbert et al., 1984), form stable drug emulsions. The colloidal stability of the emulsion particles was very good and sterile samples were stored at 4°C for several months without any aggregation or change in particle size. The chemical degradation of drugs in emulsion preparations was assessed by TLC and HPLC. No significant hydrolysis of drugs in emulsions was noted after 1 month in PBS at 4°C or after incubation in complete culture medium for 4 h at 37°C. Chemical stability is an important issue, since hydrolysis of the ester bond and conversion of the derivatives to the parent compounds would result in their release from the emulsion particles.

3.3. Cellular uptake and hydrolysis of drugs

The cellular uptake of drug solubilized in lipid emulsions and the concomitant intracellular hydrolysis of the ester bond were studied with preparations containing the labelled compound teniposide 4′-[^{14}C]oleate. Emulsion particles with the same size and emulsifiers but with the drug replaced by cholesteryl [^{14}C]oleate were used as comparison. Cholesteryl esters are, lacking serum, non-exchangeable compounds, which rules out cellular uptake of this lipid through passive surface transfer (Phillips et al., 1987). The concentration-dependent cellular uptake of drug and cholesteryl ester in emulsion droplets by K562 and T-47D cells was studied in serum-deficient medium in order to avoid interference from the lipid transfer proteins in serum. The results are presented in Fig. 3. The uptake by K562 cells (panel A) showed a similar concentration dependence for the uptake of teniposide 4′-oleate and cholesteryl oleate, however, the incorporation of drug was about 25% greater in extent than for the neutral lipid. A plausible explanation for this difference in behaviour is that part of the cellular uptake of the drug is mediated by surface transfer. This transfer process involves the desorption into and diffusion through the water interphase of the exchanging molecules. The cellular uptake of teniposide 4′-oleate and cholesteryl

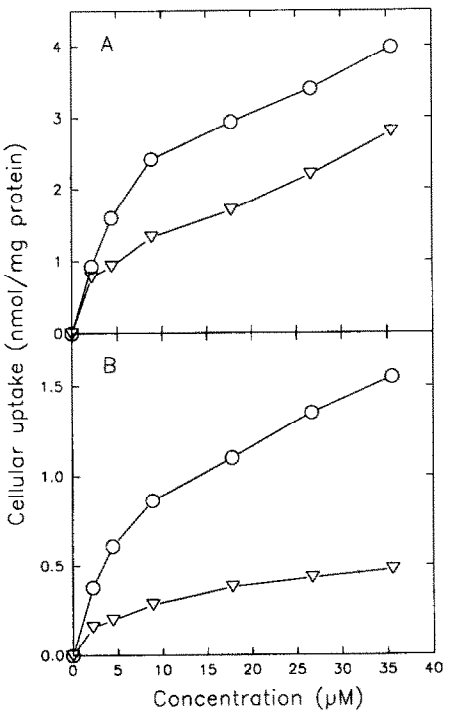

Fig. 3. Cellular uptake of teniposide 4′-[^{3}H]oleate (○) and cholesteryl [^{14}C]oleate (▽) incorporated into lipid emulsions during 4 h in serum-free medium at 37°C by K562 (A) and T-47D cells (B) in culture.

oleate by T-47D cells was less than that by K562 cells (panel B). The value obtained for the drug is in fact very similar to those determined previously for the cellular uptake of cholesterol from the same kind of lipid emulsions (Ekman and Lundberg, 1987). This observation might not be surprising, since the polarities of teniposide 4′-oleate and cholesterol are very similar as judged from the chromatographic mobility. The cellular uptake of teniposide 4′-oleate was also measured in the presence of 10% serum in the medium, however, no significant differences were obtained when compared with the results in the case of incubation in serum-free medium.

The cellular hydrolysis of teniposide 4′-[^{14}C]oleate and cholesteryl [^{14}C]oleate was measured by TLC quantitation of the labelled fatty acid liberated from these compounds. The degree of hydrolysis during a 4 h period by K562 cells was 6.3 ± 2.0 and 16.7 ± 3.0% (mean ± SD, $n = 6$) for the drug and cholesteryl ester, respectively. The corresponding values for the T-47D cells were 9.7 ± 0.6 and 28.8 ± 3.4%. The hydrolytic activity was thus greater for T-47D cells than for K562 cells. The results indicate that, after uptake, at least part of the emulsion globules, similarly to liposomes (Lundberg, 1993), end up in the lysosomes where ester bonds are hydrolyzed by acid hydrolases.

3.4. Cytotoxic activity

The antiproliferative activity of etoposide 4′-linoleate and teniposide 4′-oleate compared to that of the parent drugs was evaluated on the basis of the cellular uptake of [^{3}H]thymidine. The dose-response curves for K562 cells are depicted in Fig. 4. The linoleic acid ester of etoposide, added in the form of a drug emulsion, showed a

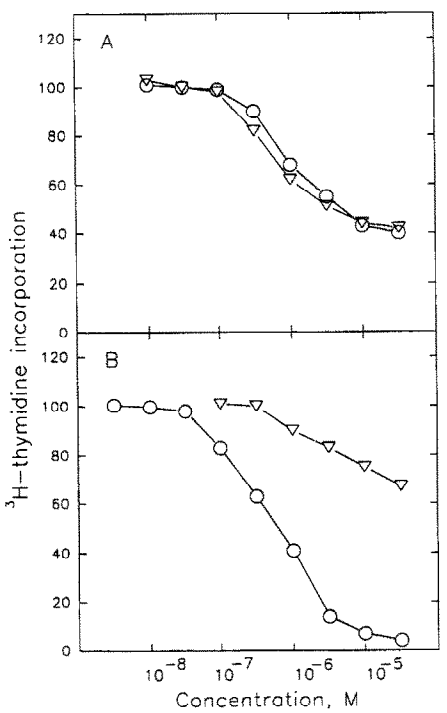

Fig. 4. Inhibition of K562 cell proliferation by drug emulsions. Control growth at 24 h = 100. (A) Cells treated with free etoposide (○) and etoposide 4′-linoleate (▽). (B) Cells treated with free teniposide (○) and teniposide 4′-oleate (▽). Each point represents the mean of four separate experiments.

somewhat higher activity than the free drug added as a DMSO-ethanol solution. In control experiments, it was shown that the emulsion preparation and the DMSO-ethanol solvent without drug did not affect the cellular uptake of [^{3}H]thymidine. For a 24 h incubation period, ID_{50} values of 3.7 and 5.6 μM were determined for etoposide 4′-linoleate and etoposide, respectively. In contrast to the relatively modest activity of the etoposide compounds, free teniposide demonstrated considerable activity against K562 cells with an ID_{50} value of 0.63 μM (Fig. 4B). Teniposide 4′-oleate, on the other hand, demonstrated low activity and 50% inhibition of [^{3}H]thymidine incorporation was not achieved during the 24 h period at the concentrations routinely used.

The cytotoxic activity of the drugs against T-47D cells is demonstrated in Fig. 5. The effects of etoposide and its fatty acid ester were very similar with a small preference for that of the free drug (Fig. 5A). The ID_{50} values of etoposide 4′-linoleate and free etoposide were 0,26 and 0,36 μg/ml, respectively. The cytotoxic activity of teniposide and its fatty acid derivative was within a smaller range than in the case regarding K562 cells (Fig. 5B). The free teniposide gave an ID_{50} value of 0.10 μM, compared to 0.70 μM for the fatty acid ester.

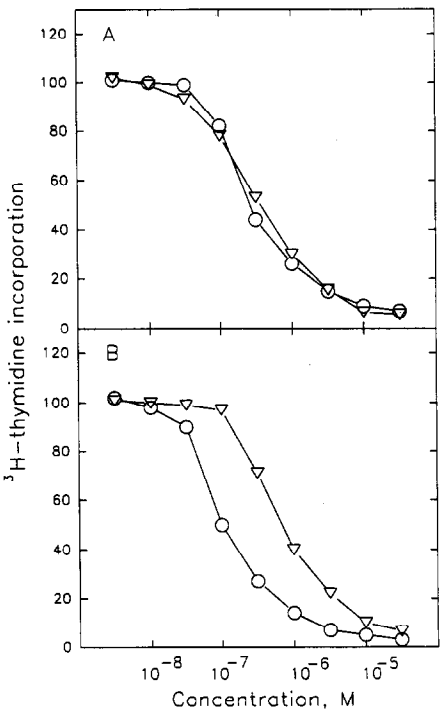

Fig. 5. Inhibition of T-47D cell proliferation by drug emulsions. Control growth at 24 h = 100. (A) Cells treated with free etoposide (○) and etoposide 4′-linoleate (▽). (B) Cells treated with free teniposide (○) and teniposide 4′-oleate (▽). Each point represent the mean of four separate experiments.

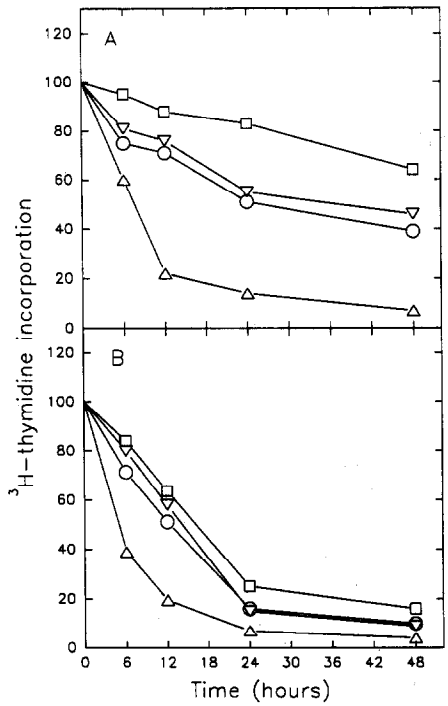

Fig. 6. Time course of cytotoxic activity against K562 (A) and T-47D cells (B) by etoposide (▽), etoposide 4′-linoleate (○), teniposide (△), and teniposide 4′-oleate (□). The concentration of the drugs was 5 μM. Data are the mean of three separate experiments.

Fig. 6 illustrates the time course of cytotoxic activity of the free drugs compared to that of the fatty acid esters. The growth inhibitory effect of teniposide initiates very rapidly and already reaches almost full effect after 12 h for both cell types. Etoposide and the both fatty acid derivatives show much slower activity which is essentially linear up to 24 h after which there is an only mild additional effect.

The growth inhibition tests clearly showed that

the fatty acid esters of etoposide and teniposide possessed cytotoxic activity in complete growth media when administered in the form of emulsions. This result differs from that of Halbert et al. (1984). The latter authors found that methotrexate-benzyl-cholesteryl diester in a lipid emulsion was inactive in normal media. Teniposide and etoposide are believed to inhibit cell growth primarily through their interaction with topoisomerase II (Yang et al., 1985). The mechanism of action is not known in detail, nevertheless, replacing the 4′-hydroxyl with a methoxy group completely inactivates the DNA cleavage activity in vivo (Loike and Horwitz, 1976). It could be rationalized that the lactone ring reacts with the enzyme while the phenolic hydroxyl at the 4′-position reacts with DNA. This hypothesis is supported by the fact that other lignans which fullfil the same structural requirements, i.e., lactone group + phenolic hydroxyl group, also inhibit topoisomerase II. From these considerations, it was assumed that the ester bond at the C-4′ position would have to be hydrolyzed before the fatty acid derivatives could exert their action. This study demonstrates a rather slow but definite intracellular hydrolysis of the fatty acid derivatives. However, the almost identical activity of etoposide and its ester is difficult to explain at this stage of the investigation.

The large difference in cytotoxic activity between teniposide and its oleic acid ester was surprising when compared to the similarity in the effects of the etoposide compounds. Etoposide is structurally identical to teniposide except for the substitution of a methyl group for the thiophene on the glucoside portion of the molecule. Teniposide is more lipophilic than etoposide, accumulates to a steady-state level about 10-times greater and is generally 10-fold more toxic (O'Dwyer et al., 1985). Regarding the cytotoxic activity of the free drugs against K562 cells, the results obtained in this study are in good agreement with the above statement. The ID_{50} value determined for etoposide is 5.6 μM as compared to 0.63 μM for teniposide. For T-47D cells the difference is less pronounced (0.36 vs 0.1 μM). Therefore, for the free drugs, the polarity of the drugs closely reflects their cytotoxic effect. However, it is not possible to apply the same criteria to the effects of the fatty acid esters. Teniposide 4′-oleate, which is more lipophilic than etoposide 4′-linoleate, still demonstrates much less activity against K562 cells and also somewhat lower activity against T-47D cells. The relatively low activity of both etoposide and etoposide 4′-linoleate as well as of teniposide 4′-oleate against K562 cells is also hard to explain. The fact that free teniposide displays high activity excludes resistance against this type of drugs. There are consequently many unresolved questions regarding the cytotoxic action of the parent podophyllotoxins as well as their fatty acid derivatives.

The major contribution of this study is that the lipophilic fatty acid esters of the two podophyllotoxins, etoposide and teniposide, can be solubilized in stable lipid emulsions and that these preparations show cytotoxic activity against cancer cells in vitro. Such lipid emulsions with a sub-micron particle size possess several favourable properties as drug carriers, e.g., they are biocompatible, biodegradable, physically stable, and easy to produce. Other assets are their large loading capacity and optical transparency which allow observation of aggregation or contamination. These sub-micron size lipid emulsions can rightly be compared to the coarse emulsions made of triglyceride oil and phospholipids used clinically for parenteral feeding. Such emulsions have also been used as carriers for lipophilic drugs (Paborji et al., 1988). They are well tolerated by the body but involve several drawbacks such as large particle size (300–400 nm) and a heterogeneous composition. In this study a mild 'biological' surfactant was used to improve the emulsification efficiency in order to reach the desired droplet diameter of about 100 nm. It is supposed that this size is the cut-off value for efficient carrier-mediated drug delivery, since it is the dimension of the largest pores in the capillary endothelium (Wisse, 1970).

This study is part of a project on the use of lipid emulsions in the nanometer range as drug carriers. An important part of this work is to develop drugs with suitable properties for incorporation into such preparations. The fatty acid derivatives of etoposide and teniposide proved to

be both lipophilic enough and sufficiently oil soluble for this purpose. The long-term goal of the project is to produce carrier systems with targeting capacity where ligands for cellular receptors are attached to the surface of the emulsion droplets. To some extent, this goal has already been attained. In previous papers from this laboratory, a steroid mustard carbamate (Lundberg, 1987) and prednimustine (Lundberg, 1992) were solubilized in lipid emulsions and then complexed with apolipoprotein B, which is recognized by the low-density lipoprotein receptor.

Acknowledgments

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