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TENIPOSIDE-INDUCED CHANGES IN THE PHYSICAL PROPERTIES OF PHOSPHATIDYLCHOLINE LIPOSOMES

A CALORIMETRIC STUDY

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Abstract—The anticancer agent teniposide has significant effects on plasma membrane components, in addition to its well known nuclear effects. Alterations in the properties and function of cellular membranes by various amphipathic compounds have been attributed previously to their relatively non-specific interactions with membrane components. We have examined the interaction of teniposide with defined model membranes by monitoring drug-induced changes in the melting profile of phospholipids by differential scanning calorimetry. The main phase transition temperature of dimyristoyl- or dipalmitoylphosphatidylcholine was lowered and broadened by the presence of teniposide in the liposomes. These effects were essentially linear over the concentration range of 1–5 mole %. The calorimetric enthalpy of the gel to liquid-crystalline transition of the phospholipids was not changed by the addition of the drug. The characteristic pretransition of these saturated phospholipids was decreased by teniposide concentrations as low as 0.1 mole % and was abolished at teniposide concentrations greater than 1 mole %. The data confirm the lipophilic nature of teniposide and indicate that the non-specific interactions with membrane lipids should be considered when evaluating the membrane-related effects of this agent.

The semisynthetic epipodophyllotoxin glucopyranosides are a relatively new class of anticancer agent that has been shown to be active against a variety of leukemias and solid tumors [1]. Teniposide (VM-26, 4′-demethyl epipodophyllotoxin thenylidene-β-D-glucoside) is the most potent drug within this group of semisynthetic agents. Teniposide is a derivative of the naturally occurring podophyllotoxin, but unlike the parent compound it has no effect on tubulin or microtubules [2]. Teniposide is primarily cytotoxic through its interactions with DNA and associated enzymes [3]. Although the nuclear effects appear to be the primary determinant of cytotoxicity, one of the first noted biochemical effects was inhibition of the facilitated transport of nucleosides across the plasma membrane [2]. This membrane-related activity of the epipodophyllotoxins has since been confirmed for transport of nucleosides [4] and of therapeutic nucleoside analogs [5]. In addition, efflux of methotrexate via the energy-dependent folate carrier has been shown to be inhibited by teniposide [6]. Since such apparently diverse transport systems appear to be sensitive to these agents, their inhibitory effects may be due to a more generalized membrane perturbation.

Many small amphipathic compounds are known to elicit not-specific membrane perturbing effects following their insertion into the lipid bilayer [7, 8]. The interaction of amphipathic drugs with the lipid phase of membranes results in alterations in the physico-chemical properties of the lipids. Changes in the phase transition temperature, fluidity, and permeability of drug-containing liposomes have been reported which indicate changes in the dynamic properties of lipids [7–9]. Because the activity of most integral membrane depends on specific lipids, including that protein which mediates nucleoside transport [10], direct association between drug and lipid may account for the changes in activity of the membrane proteins. Recently, membranes of the cell surface have been suggested as a potential target through which a variety of chemotherapeutic agents may be cytotoxic [9].

Etoposide (VP-16), a similar but more hydrophilic congener of teniposide, has been examined previously for its affects on membrane lipid properties [11]. In this preliminary study, etoposide was shown to alter the phase transition profile and to act as a fusagen for a mixed lipid system [11].

The aim of the present work was to examine by physical methods the interactions of teniposide with defined lipids so that its interaction with membranes may be more fully understood. Analysis of the thermotropic transitions of membrane lipids by differential scanning calorimetry (DSC) has been utilized as a fundamental technique to detect the presence of any compounds which have penetrated into the lipid domain. DSC was employed in the present study to examine the ability of teniposide to interact with lipid bilayers. The saturated phospholipids dimyristoyl- and dipalmitoylphosphatidylcholine were chosen as model membranes for this study because of their well characterized phase behavior which includes a low enthalpy pretransition. Utilizing lipids exhibiting a pretransition

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increases the sensitivity of the method since the pretransition may be a more sensitive indicator of drug–lipid interactions than the main transition [8].

The plasma membrane is the initial site of interaction between drug and cell as the drug diffuses toward its nuclear target. We believe that these earliest membrane effects are important for they may contribute to the cytotoxicity of teniposide. Perhaps more importantly, perturbation of the plasma membrane structure by teniposide may alter the permeability for other chemotherapeutic agents, thereby altering the desired enhancement of cell kill by combination chemotherapy.

MATERIALS AND METHODS

Dipalmitoyl L-α-phosphatidylcholine (DPPC) (lot 010163) and dimyristoyl L-α-phosphatidylcholine (DMPC) (lot 810031) were purchased from Calbiochem, San Diego, CA. Teniposide was obtained from Bristol Laboratories, Syracuse, NY.

Lipids migrated as a single spot by thin-layer chromatography and were used without further purification. Purity of the lipids was also evident by the narrow phase transition profile. Teniposide and lipids were stored in chloroform/methanol (2/1) in the dark at −20°. Appropriate amounts of teniposide (0.1–5 mole %, 6.1–330 μg) were combined with 7 mg of DPPC or DMPC and evaporated to dryness under a stream of argon. Residual solvent was removed by vacuum desiccation overnight at room temperature. Dry lipids were hydrated by the addition of 100 μl of 10 mM 4-(2(hydroxyethyl)-1-piperizine-ethane-sulfonic acid (HEPES) (pH 7.4) at a temperature 5–10° above the phase transition temperature. The lipids were dispersed by vortex mixing and maintained above the transition temperature for 1 hr prior to calorimetry. Liposomes generated by this method are known to form multilamellar structures [12]. For DSC measurements, 75 μl of lipid suspension containing 5 mg of phospholipid was hermetically sealed in aluminum pans and placed in a DuPont 1090 differential scanning calorimeter. Indium and gallium standards were used for instrument calibration. Samples were heated at a rate of 1°/min. The main phase transition temperature, T_m, was defined as the peak of the gel to liquid-crystalline endotherm. The van't Hoff enthalpy, an indicator of the sharpness of the transition, was determined as:

$$\Delta H_{vH} = 6.9\,(T_m 2/WHH)$$

where *WHH* is the width at half-height of the transition profile. Cooperative unit sizes were determined from the ratio of van't Hoff to calorimetric enthalpies [13]. Data were subjected to Student's paired *t*-test for statistical analysis. Lipid phosphorus was assayed on all samples following calorimetry by the method of Chalvardjian and Rudnicki [14] to validate the quantitative delivery of lipid to the sample pans.

RESULTS

DPPC liposomes. The effect of teniposide on the physical properties of model membranes was examined by monitoring the melting behavior of DPPC liposomes in the presence of the drug. Figure 1 represents a series of DSC heating scans of DPPC in the presence of increasing amounts of teniposide. Each trace is that of a typical DSC scan and represents the change in excess specific heat for the transition from gel to liquid-crystalline phase during the heating cycle. The temperature for the main chain melting transition was 41.6° for DPPC. Saturated phosphatidylcholines undergo a pretransition from a solid phase to an intermediate phase in which the lipid lamellae is distorted by a periodic ripple [15]. This pretransition occurred at 36.4° for untreated DPPC. Addition of teniposide resulted in profound changes in the thermotropic behaviour of the lipid. Addition of as little as 0.1 mole % drug resulted in a downward shift of 0.7° for the pretransition. No evidence of a pretransition was noted at teniposide concentrations in excess of 1 mole %. The temperature of the main transition was progressively decreased (correlation coefficient = 0.966) by the addition of teniposide, with 5 mole % drug lowering the transition by 1.4°. The calorimetric enthalpy (ΔH) for DPPC was 8.44 kcal/mole. No change in ΔH was observed in the presence of teniposide at any of the concentrations used (Table 1). A decrease in ΔH would indicate that the total number of lipids undergoing the transition would be effectively decreased. This lack of effect by teniposide on ΔH suggests that there is not a tight binding of lipid with drug. Since the transition was progressively broadened by the addition of teniposide, the calculated van't Hoff enthalpy was decreased proportionately. A comparison of the van't Hoff and calorimetric enthalpies can be used as a measure of the intermolecular cooperativity of the transition process for a given set of lipids. The

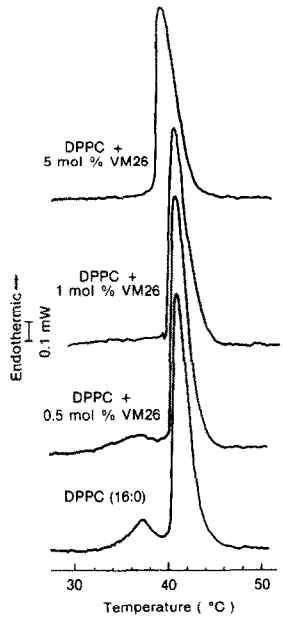

Fig. 1. Thermograms of DPPC liposomes incubated with teniposide as described in Materials and Methods.

Table 1. Thermodynamic parameters of DPPC in the presence of teniposide*

Teniposide concentration (mole %)	T pre† (°C)	T main‡ (°C)	Transition half-width§ (°C)	ΔH‖ (kcal/mole)
0	36.4 ± 0.6	41.6 ± 0.2	1.8	8.44 ± 1.03
0.1	35.7 ± 0.7	41.6 ± 0.3	1.8	7.56 ± 0.78
0.5	34.9 ± 0.6¶	41.6 ± 0.2	1.8	8.36 ± 0.60
1	ND**	41.4 ± 0.3	2.0	8.23 ± 0.47
3	ND	41.1 ± 0.1¶	2.6	7.73 ± 0.51
5	ND	40.2 ± 0.8¶	2.6	8.14 ± 0.32

* Values represent the mean of three to five independent experiments ± S.D.
† Temperature of the pretransition peak.
‡ Temperature of the main or chain-melting transition peak.
§ Width at half-height of the main transition peak.
‖ Calorimetric enthalpy of the main transition.
¶ Significantly different from pure DPPC at the $P < 0.05$ level.
** Pre-transition not detectable.

cooperative unit size is an indication of the number of lipid molecules melting as a group within the bilayer and has a theoretical value of infinity for an absolutely pure preparation undergoing a first order transition [13]. Since it is very sensitive to even trace impurities, the cooperative unit size varies among lipid preparations; however, relative changes in the cooperative unit size are useful for evaluating the presence of contaminants, such as teniposide in this case. In the presence of 5 mole % teniposide, the size of the cooperative unit is decreased by 26% ($P < 0.05$), indicating that integration of the drug into the bilayer is changing the interactions between phospholipids within the bilayer.

DMPC liposomes. The thermotropic properties of DMPC are quantitatively different from those of DPPC due to the differences in fatty acid chain lengths; however, the effects of teniposide addition were similar for the two phospholipids examined.

The main transition temperature for DMPC was 23.1° and was decreased by 1.3° with the addition of 5 mole % teniposide (Fig. 2). The effects on the main transition were linear (correlation coefficient = 0.998) over the concentration range of 0–5% examined. The cooperativity of the transition from gel to liquid-crystalline phase was also altered significantly; the peak width at half-height was doubled by the addition of 5 mole % teniposide. The pretransition, centered at 12.8° for DMPC, was again abolished by drug concentrations above 1 mole %.

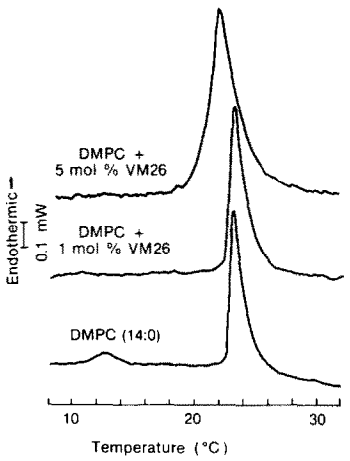

Fig. 2. Thermograms of DMPC liposomes incubated with teniposide as described in Materials and Methods.

DISCUSSION

Disaturated phosphatidylcholine liposomes undergo reversible, highly cooperative phase transitions that can be detected by several physical techniques including DSC. The temperature at which the gel to liquid-crystalline transition occurs is dependent upon both the nature of the head group and the fatty acid composition of the phospholipid. The lipid phase transition temperature is also very sensitive to the presence of foreign molecules which have partitioned into the bilayer. Therefore, DSC of defined lipid systems has been utilized extensively as a technique to physically establish the presence of amphipathic drugs within a lipid membrane [16]. In the current study, graded amounts of the anticancer agent teniposide were mixed with DMPC or DPPC, and their phase behavior was followed by DSC. Addition of teniposide from 1 to 5 mole % progressively broadened and lowered the phase transition of either disaturated species. This drug-induced perturbation of the thermotropic phase transition is taken as evidence for the insertion and integration of teniposide into the multilamellar lipid structures. In addition, the characteristics of the altered phase transition may be used to estimate where the drug may reside within the bilayer. Reduced transition temperatures and broadened transition peaks are characteristic of bilayers modified by agents which are believed to intercalate into the C(2)–C(8) region of the fatty acyl chains of the phospholipids [16]. Raman spectroscopy has confirmed that drugs which induce this type of phase change do not perturb either the headgroup or glycerol backbone regions of DPPC bilayers [8]. Since teniposide apparently interacts primarily with the hydrophobic acyl portion of the bilayer, it should show little specificity for the head group. The affinity

of teniposide for lipids should therefore be relatively independent of the head group of the phospholipid.

Linear regression analysis of the change in T_m versus teniposide concentration for DPPC indicates that 4 mole % drug would result in a 1° decrease in the T_m. This result is in close agreement with the 4.3% predicted for a 1° depression when the determined ΔH (8.44 kcal/mole) and T_m (41.6°) for DPPC are integrated into a modified van't Hoff equation [17]. The concentrations of teniposide reported here are ratios of those that were co-deposited with the lipid and may not reflect actual drug concentrations within the membrane. However, the close correlation between the observed and predicted effects on the phase transition suggests that most of the added drug is incorporated into the membrane. If a significant portion of the drug indeed remains in the aqueous phase, then teniposide may be even more potent than our data suggest.

The effects of teniposide on the main transition were similar to those previously noted for a variety of dissimilar agents which are related only in their abilities to perturb membranes [8, 9, 16, 17]. For example, medium chain length *n*-alkanols reduce the transition temperature by 1° at concentrations of 4.4 mole % [17]. Steroidal and non-steroidal anesthetics and chemotherapeutic agents have been shown to alter the DSC profile for many different types of lipids [8, 9, 16]. In addition, these compounds display pronounced variations in potency as a result of only small structural differences. Since we have shown that teniposide interacts with membrane lipids, it is possible that it may also act as a non-specific membrane perturbant that indirectly alters the function of lipid-sensitive membrane proteins via its interaction with membrane lipids. Once integrated into the lipid domain, the drugs should have lateral diffusion coefficients on a time scale similar to that of the lipids with which they are associated. We believe that lateral movement into protein rich domains could account for the inhibition of membrane transport systems by the epipodophyllotoxins [2, 4–6]. Alternatively, the drug may influence membrane function by changing the bulk properties such as membrane fluidity or permeability [11].

White *et al.* [5] have reported potent inhibition of ara-C transport in Ehrlich ascites tumor cells by teniposide. Addition of 50 μM teniposide to Ehrlich cells suspended at a density of 2×10^7 cells/ml inhibited transport by 88%. Ehrlich cells contain approximately 80 fmoles of lipid phosphorous/cell (unpublished data) so that, at the cell density used for the transport experiments, the total cellular lipid concentration is 1.6 mM. Thus, the molar ratio of teniposide to phospholipid was approximately 3%. Although comparison of the effects of teniposide on defined lipid systems with cellular membranes must be made with caution, these calculations suggest that the biophysical effects of teniposide on liposomes at 1–5 mole % may be relevant to its biochemical effects on intact cells.

The greater inhibition of ara-C and methotrexate transport by teniposide over etoposide has been attributed to a greater oil solubility of the former [5, 6]. The oil/water partition coefficients for the epipodophyllotoxins are 7.2 and 2.7 for teniposide and etoposide respectively [18]. Since the degree of membrane perturbation may be expected to correlate with the oil/water partition coefficient for the agent, etoposide at equimolar concentrations should alter the lipid phase transitions to a lesser extent than teniposide. In preliminary studies with etoposide, we have indeed noted lesser effects on the lipid phase transition when compared with teniposide [19]. This is in agreement with the qualitative studies of Koehler *et al.* [11] who initially reported that etoposide lowered and broadened the lipid phase transition of a mixed lipid system.

An estimation of the membrane/buffer partition coefficient of teniposide based upon an analysis of the DSC data by the method of Kamaya *et al.* [20] yields values 1–2 orders of magnitude greater than the reported oil/water partition coefficients [18]. However, this empirical treatment requires ideal mixing of the solute within the bilayer. Other agents which are thought to be localized in the C(2) to C(8) methylene region of the bilayer, such as the medium chain length *n*-alkanols [16], have been shown recently to form clusters rather than being randomly distributed within the bilayer [21]. If teniposide and agents with similar structures do cluster within the bilayer, then these formations may represent a type of lateral phase separation which is believed to be present in lipid mixtures and in biomembranes [22].

The pretransition which is exhibited by only a select group of phospholipids (disaturated species of phosphatidylcholine, phosphatidylglycerol, and cardiolipin) is known to be abolished by small additions of cholesterol, fatty acids, or other amphipathic agents [8, 9, 13, 16]. A hydration-dependent thermotropic lattice rearrangement into a "ripple" phase characterizes the pretransition [15]. This pretransition, although poorly understood, always occurs at lower temperatures and has a lower enthalpy of transition than the main transition which involves transient trans/gauche isomerizations within the fatty acyl chains [15]. The pretransitions of both DMPC and DPPC were altered at concentrations of teniposide well below those which induced observable changes in the main transition profile. The greater sensitivity of this early transition to contaminants such as teniposide could be accounted for by the more subtle molecular changes that characterize the lower energy phase change.

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