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Partitioning of teniposide into membranes and the role of lipid composition

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We have examined the partitioning behavior of the anticancer agent teniposide (VM-26) into multilamellar vesicles composed of various phospholipid species. Partitioning was found to be sensitive to the composition of the liposomal membrane since changes in the head group or acyl chain constituents could dramatically alter the affinity of the drug for the bilayer. [^{3}H]VM-26 partitioned most readily into 1,2-monounsaturated species of phosphatidylcholine (PC) with a molar partition coefficient (K_p) of 4290 for dioleoyl-PC at 37°C. Inclusion of additional phospholipids having a different head group reduced partitioning in the order cardiolipin > phosphatidylglycerol > phosphatidylserine > phosphatidylethanolamine. The K_p for dioleoyl-PC with 33 mol% cardiolipin was reduced to 1370. Partitioning into completely saturated species of PC was much less than that for unsaturated species and was inversely proportional to the hydrocarbon chain length at temperatures either above or below the chain melting temperature. The K_p for fluid phase dimyristoyl-PC was 2300. Partitioning into dimyristoyl-PC or dioleoyl-PC at 37°C (fluid) or dipalmitoyl-PC at 25°C (gel) was reduced by the addition of 5–30 mol% cholesterol in proportion to its bilayer concentration. Etoposide (VP-16) at concentrations up to 10 mol% did not compete with [^{3}H]VM-26 for association with dioleoyl-PC. Addition of calf serum or serum albumin could significantly reduce the association of [^{3}H]VM-26 with the liposomes.

Introduction

The epipodophyllotoxin glucopyranosides are a group of semisynthetic podophyllotoxin derivatives which have been found to be potent anticancer agents. Efficacy for clinical use in the treatment of a variety of neoplasms has been established for two of these derivatives, teniposide (VM-26) and etoposide (VP-16) [1]. These compounds are believed to inhibit cell growth primarily through their interaction with topoisomerase II [2,3] although they exhibit a broad range of biological activity which includes many membrane-related effects [4–8]. Among the membrane-associated processes which are sensitive to these agents are carrier proteins which mediate the transport of other drugs which are often co-administered. These include the carriers for antifolates such as methotrexate [4] and for nucleoside analogs such as ara-C [5]. These potential adverse actions on drug transport are important since the growth-inhibitory effects of the latter agents depend upon rapid uptake and processing by the tumor cells.

Many of the membrane-associated effects of epipodophyllotoxins are believed to be mediated by their ability to interact with membrane lipids. We have previously demonstrated that the epipodophyllotoxins can alter the fluidity of membranes of Ehrlich and CEM-CCRF cells nd of lipid vesicles composed of natural and synthetic lipids [7]. In the studies with lipid vesicles, VM-26 was found to increase acyl chain order in bilayers composed of saturated phosphatidylcholine (PC) more extensively than those composed of mixed soy lipids or cholesterol-containing membranes. In addition, although VM-26 lowered the chain melting temperature of dimyristoyl-PC (DMPC) and dipalmitoyl-PC (DPPC) by similar magnitudes [8], the effects on acyl chain

Abbreviations: VM-26, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidine-β-D-glucopyranoside), teniposide; VP-16, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-ethylidene-β-D-glucopyranoside), etoposide; K_p, partition coefficient; DPH, 1,6-diphenylhexatriene; ESR, electron spin resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; CL, cardiolipin (CL was from bovine heart and contains predominantly 18:2); DL, dilauroyl-; DM, dimyristoyl-; DPO, dipalmitoleoyl-; DP, dipalmitoyl-; DPD, dipentadecanoyl-; DS, distearoyl-; DO, dioleoyl-; PO, 1-palmitoyl-2-oleoyl-; CMC, critical micellar concentration.

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mobility was more pronounced for DMPC [7]. These observations suggest that VM-26 may have a preferential interaction with specific lipid species. In the current studies, we have constructed model membranes of varied composition and have investigated the bilayer properties important for association of VM-26 with lipid membranes.

Materials and Methods

Chemicals. The epipodophyllotoxins VM-26 (4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidine-β-D-glucopyranoside); teniposide) and VP-16 (4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-ethylidene-β-D-glucopyranoside); etoposide) were obtained from the Pharmaceutical Research and Development Branch of Bristol Myers Co. 1,6-Diphenylhexatriene (DPH) was purchased from Aldrich. Phospholipids were obtained from Avanti Polar Lipids and used without further purification. Lipid purity was checked periodically by TLC. Cholesterol and 4-(2-hydroxyethyl)-1-piperizine-ethanesulphonic acid (Hepes) were obtained from Sigma. [^{14}C]Inulin was purchased from ICN and purified on Sephadex G-75. [^{3}H]VP-16 (480 mCi/mmol, Moravek) was a gift from Dr. Jack Yalowich (St. Jude's Childrens Hosp., Memphis, TN). [^{3}H]VM-26 was prepared from nonlabeled VM-26 by catalytic tritium exchange with [^{3}H]$_2$O in an organic solvent by Amersham Radiochemical Co. The labeled material was purified by HPLC and thin-layer chromatography (TLC). Silica gel G TLC rediplates were developed in $CHCl_3$/MeOH/H_2O/NH_4OH (70 : 30 : 2 : 3, v/v). The R_F for VM-26 was 0.85. [^{3}H]VM-26 was extracted from the silica gel with ethyl acetate. After evaporation of the solvent, the product was dissolved in DMSO/EtOH (1 : 1, v/v) and stored at −20 °C. The product remained radiochemically pure for at least two months.

Determination of CMC. Apparent critical micellar concentrations for VM-26 was determined by right-angle light scattering. The incident beam was from a 6 mW He-Ne laser (633 nm, Spectra Physics Model 120) and light was detected at 90° with a red-sensitive R928P photomultiplier tube (Hamamatso) and digital photometer (Pacific Precision Instruments) which were interfaced to an Apple IIe computer with a Laboratory Interface Peripheral System (Research Digital Analog) capable of collecting data at the rate of 10 points/s. Samples were filtered through 0.2 μm filters just prior to analysis.

Liposome preparation. Chloroform or chloroform/methanol solutions of lipid and labeled drug were co-deposited in 12 × 75 mm glass tubes and dried down with nitrogen. Residual solvent was removed by vacuum desiccation for at least one hour. Multilamellar vesicles (liposomes) were formed by hydration with 10 mM Hepes (pH 7.4) at a temperature at least ten degrees above the chain-melting temperature (T_c) of the highest melting component. Lipids were dispersed by vortex mixing. Unless otherwise indicated, the total lipid concentration was 3 mM and the concentration of [^{3}H]VM-26 was kept at less than 0.1 mol%

Partition measurements. Membrane/buffer partitioning was determined from the distribution of radiolabel between liposomes and buffer following their separation by high-speed centrifugation. Following hydration and dispersion the samples were incubated at the desired temperature for 2 to 4 h and then aliquoted into 0.2 ml cellulose propionate airfuge tubes (Beckman). The samples were then centrifuged with a Beckman airfuge at 130 000 × *g* for 10 min at 25 °C. Greater than 95% of the lipid is pelleted under these conditions. The clear supernatant was removed and sampled for radioactive counting and the lipid pellet was dissolved in 0.2 ml of 0.5–1% Triton X-100. The solubilized lipid and two water washes of the airfuge tube were combined and counted for radioactivity. Parallel experiments were performed with [^{3}H]inulin to permit correction for trapped aqueous [^{3}H]VM-26 in the pellet. The partition coefficient (K_p) was calculated on a molar basis (*a*) where:

$$K_p = \frac{\text{mol of drug in lipid/volume of lipid}}{\text{mol of drug in buffer/volume of buffer}}$$

Lipid density was taken to be 1 g/ml.

Anistropy measurements. Steady-state fluorescence anisotropy measurements of 1,6-diphenylhexatriene were performed on a Perkin-Elmer LS-5 spectrofluorometer as described previously with minor exceptions [23]. The lipid/probe ratio was 500 : 1 with a lipid concentration of 0.3 mM. DPPC samples were heated to 50 °C for 3 h and fluorescence measurements were made the following day. Data are presented as anisotropy (*r*) values calculated as before [23].

Results

Solubility of VM-26

The epipodophyllotoxins are known to be poorly water soluble which necessitates the use of various detergents and solubilizers in their pharmaceutical formulations. To evaluate more precisely the physicochemical properties of VM-26 we employed dynamic laser light scattering to determine the concentration at which the drug aggregates and becomes water insoluble. Scattered light intensity as a function of VM-26 concentration indicated an apparent CMC of 52 μg/ml (78 μM) in water (Fig. 1). At concentrations greater than this, large micelles or aggregates of drug formed which had a high propensity to scatter incident light as compared to the monomers. The molecular weight of VM-26 monomers is 656 whereas that of the aggregates formed was estimated to be greater than 10^6 which indicates an aggregation number in excess of 1500.

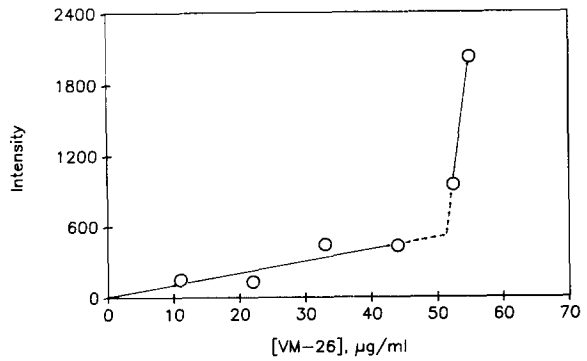

Fig. 1. Stock VM-26 (25 mM suspended in DMSO/ethanol, 1:1) was diluted in water to the indicated concentration. Right-angle light scattering intensity was determined as described in Methods. The intercept of the two intersecting lines was determined by linear regression analysis to be 78 μM.

Concentration-dependence of partitioning

The very high propensity of the drug to self associate into large hydrophobic assemblies suggests that the drug will also prefer a hydrophobic lipid domain over an aqueous environment. To determine the amount of VM-26 which could be effectively incorporated into lipid membranes, the concentration dependence of partitioning was examined. Partitioning of [^{3}H]VM-26 into multilamellar vesicles of DOPC was determined at 37 ° C as a function of the amount of unlabeled VM-26 added. As seen in Fig. 2 addition of VM-26 at concentrations greater than 1–2 mol% proportionally reduced the partition coefficient for [^{3}H]VM-26. There was a linear decrease in partitioning with increasing drug incorporation. At a total drug concentration of 26 mol% partitioning of any additional drug should be negligible, as determined from the *X*-intercept of Fig. 2. This suggests that the lipid phase can be saturated with drug under these conditions.

Although the concentration of [^{3}H]VM-26 in all of these studies was maintained at less than 0.1 mol% relative to lipid, when unlabeled drug was added the total drug concentration at 3 mol% and above was in excess of the CMC of free VM-26. To examine the potential relationship between K_p and CMC we evaluated partitioning at lower lipid and total drug concentrations. The results indicated that partitioning appears to progressively drop off upon addition of unlabeled VM-26 in excess of 2–3 mol% (Fig. 2) even though under these conditions the CMC is exceeded only at concentrations greater than 8 mol%. This suggests that the drug probably has no greater tendency to self-associate in aggregates than it has for association with lipids. The apparent saturation point at 2 mol% derived from these two sets of data for DOPC indicate that a lipid/drug ratio of 50:1 (mol:mol) can be obtained before partitioning starts to drop off.

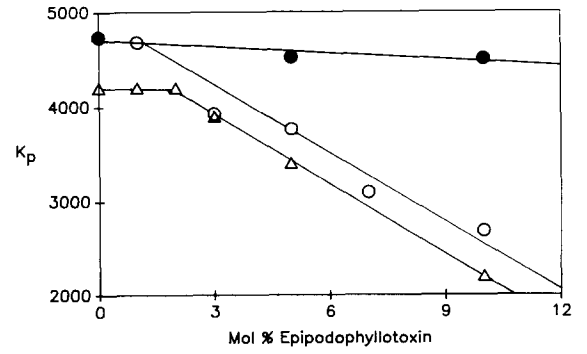

Fig. 2. Partitioning as a function of drug concentration. Partitioning of [^{3}H]VM-26 into DOPC vesicles was measured at 37 ° C. Unlabeled VM-26 (○,△) or VP-16 (●) was added to lipid at the indicated mol ratio at a total concentration of drug+lipid of 3 mM (○,●) or 1 mM (△).

We have previously demonstrated that VM-26 concentrations of 2 mol% and above can perturb PC bilayers in a concentration-dependent manner [7,8]. The reduced accumulation of [^{3}H]VM-26 in the presence of at least 20-fold excess unlabeled drug may therefore be due to either saturation of lipid with drug, or alternatively, due to a less favorable membrane environment which has been altered in the presence of the drug.

A reduced association with lipids for drugs as their concentration is increased has been reported for other amphipaths including amphotericin B [10], chlorpromazine [9,11,12] various chlorinated hydrocarbons [13], and benzene [22]. Interestingly, lipid partitioning of chlorpromazine, which has a CMC close to that of VM-26, also starts to drop off when the total concentration exceeds 2 mol% [11] to 4 mol% [9].

VP-16 is a related compound which is structurally identical to VM-26 except for the substitution of a methyl group for the thiophene on the glucoside portion of the molecule [1]. This simple alteration significantly changes the polarity of the compound which is evident in the octanol/water partition coefficients which are 44 for VM-26 and 6 for VP-16 [14]. These differences are also reflected in cellular uptake where it has been shown that VP-16 accumulates to steady-state levels only one-sixth to one-tenth that of VM-26 [15,16]. VP-16 is generally 10-fold less cytotoxic than VM-26 [1], binds to albumin less avidly [16], and also has reduced potency for perturbing membranes [5,7]. Although VP-16 is significantly more hydrophilic than VM-26 it too can associate with lipids and, to a lesser extent, alter the phase transition temperature [8] and acyl chain mobility of membranes [7].

To determine if these two congeners compete for similar sites within the bilayer we have examined [^{3}H]VM-26 partitioning in the presence of unlabeled VP-16. Addition of up to 10 mol% VP-16 to DOPC had no effect on [^{3}H]VM-26 accumulation (Fig. 2). This indicates that either VP-16 does not associate with DOPC or that the two drugs do not compete for common sites within the membrane. Since we have previ-

TABLE I

Role of lipid head group on partitioning of teniposide into phospholipid mixtures

Partitioning was evaluated at 37°C as described in Methods. The partition coefficient (K_p) is presented as the mean ± S.E.

Lipid	Mole ratio	K_p
DOPC	–	4288 ± 206
DOPC/DOPE	2:1	2275 ± 108
DOPC/DOPS	2:1	2132 ± 274
DOPC/DOPG	2:1	1478 ± 195
DOPC/CL	2:1	1368 ± 29
POPC	–	2817 ± 243
POPC/POPE	1:1	1165 ± 60

ously demonstrated the ability of VP-16 to intercalate into vesicles of disaturated PC species we believe that both drugs are actually in the membrane but at different locations. These results may explain in part the inability of VM-26 to reduce VP-16 accumulation in L1210 cells reported by Allen [15].

Role of head group

We had previously observed stronger bilayer perturbing effects of VM-26 on membranes composed of pure PC than for mixed lipid membranes such as soy asolectin which is composed primarily of PC and PE. To evaluate the role of lipid head group in membrane partitioning of VM-26 we utilized binary lipid mixtures composed of PC and one of four other phospholipids. Preferential association was demonstrated for PC since inclusion of any alternate species into DOPC reduced the association of VM-26 with the membranes (Table I). For liposomes constructed of DOPC and another neutral or acidic lipid at a molar ratio of 2:1, partitioning decreased in the order DOPE > DOPS > DOPG > cardiolipin. Inclusion of 33 mol% cardiolipin reduced VM-26 partitioning to one-third of the value for pure PC. Liposomes composed of 2:1 DOPC/DOPE incorporated 47% less drug than for DOPC alone and decreasing the concentration of PC further (by 17%) reduced partitioning (by 12%) into similar mixtures of POPC and POPE (1:1). Since the order of effectiveness of the various lipids to reduce partitioning parallels their net charge [17] this suggests that association of the drug with the liposomes may be due in part to ionic interactions. However, when partitioning into DOPC was examined as a function of the proton concentration in the pH range of 8 to 4 the ability of the drug to associate with the membrane was not altered (data not shown). Furthermore, addition of 30 mM $CaCl_2$ did not affect partitioning. Octanol/water partitioning of VM-26 has previously been reported to be relatively pH-independent [18]. This suggests that the effects are due to the characteristics of the bilayer imparted by these polar lipids rather than direct ionic interactions between drug and lipid.

Role of acyl chain

To further evaluate the bilayer physical constraints influencing partitioning of VM-26, the sensitivity of partitioning to variations in acyl chain composition and structure was examined. While keeping constant the phosphorylcholine head group, the fatty acids for the 1,2-diacyl species were varied in terms of chain length and degree of saturation. Partitioning was highest for the monounsaturated species with dipalmitoleoyl-PC (16:1) accumulating the greatest amount of drug with a K_p of 5052 (Table II). In contrast, partitioning into the saturated species (DPPC) having the same chain length (16:0) and measured at the same temperature (37°C) was decreased 85% as compared to the monounsaturate. An even larger difference was noted for 18:1 vs. 18:0 at 37°C. With the five disaturated species of PC tested, partitioning was found to be inversely proportional to the chain length at a temperature which was either above or below their respective chain melting temperature (Fig. 3). Moreover, a sharp increase in drug-lipid association was found for liposomes which were in the liquid-crystalline or fluid phase as compared to lipids in the gel or solid phase at the same temperature.

Since membrane fluidity, specifically acyl chain mobility, is also decreased with increasing chain length and with degree of hydrocarbon saturation, a potential relationship between partitioning and fluidity was evident. In addition, we have demonstrated previously that VM-26 itself can also decrease acyl chain mobility [7] which may account for the reduced partitioning as the unlabeled drug concentration was increased. Although

TABLE II

Fatty acyl chain dependence of teniposide partitioning into phosphatidylcholine

Partitioning was evaluated at 37°C as described in Methods. K_p represents the mean ± S.E.

Lipid	Acyl chain	Phase	K_p
DLPC	12:0/12:0	fluid	2670 ± 128
DMPC	14:0/14:0	fluid	2305 ± 91
DPDPC	15:0/15:0	fluid	1868 ± 12
DPPC	16:0/16:0	ripple	766 ± 28
DPOPC	16:1/16:1	fluid	5052 ± 339
DSPC	18:0/18:0	gel	191 ± 37
POPC	16:0/18:1	fluid	2817 ± 243
DOPC	18:1/18:1	fluid	4288 ± 206
Egg PC	mixed	fluid	2735 ± 182
Mixtures			
DMPC/DSPC	di14:0/di18:0	mixed	1604 ± (127)
DLPC/DPPC	di12:0/di16:0	mixed	2081 ± (128)
DLPC/DPPC/Chol (45:45:10)			1448 ± (85)

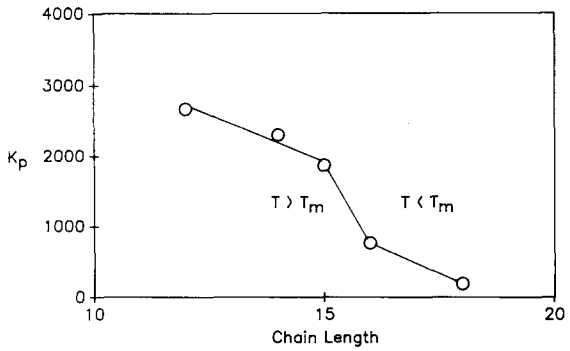

Fig. 3. Effect of acyl chain length on partitioning. Partitioning into disaturated species of PC was measured at 37 °C which was above the phase transition of DLPC, DMPC and DPDPC and below that of DPPC and DSPC.

these results suggested that partitioning was sensitive to membrane fluidity, modulation of this parameter by other means indicated that there was actually little or no correlation between [^{3}H]VM-26 partitioning and fluidity of the bilayer. Fluidity could be manipulated with either temperature adjustments or by inclusion of cholesterol which is thought to fluidize gel-phase lipids and to rigidify fluid lipids. These effects of cholesterol were verified in studies in which 5–30 mol% cholesterol and 0.2 mol% 1,6-diphenylhexatriene (DPH) were incorporated into DPPC or DOPC. Fluorescence anisotropy of DPH was then monitored under conditions in which DPPC was in the gel phase (25 °C, T_c = 41 °C) and DOPC was in the fluid phase (25 °C, T_c < 0 °C). In either system there was a linear effect of cholesterol on molecular order; increasing anisotropy in DOPC and decreasing it in DPPC (Fig. 4a). However, VM-26 partitioning into DOPC, DPPC, or DMPC was inhibited by cholesterol under similar conditions (Fig. 4b, c). The cholesterol concentration at which partitioning would be negligible (X-intercept) is 31 mol% for DPPC and DMPC and 51 mol% for DOPC.

The results of experiments designed to examine the temperature sensitivity of partitioning into DOPC and DPPC are presented in Fig. 5. The phase sensitivity of partitioning into DPPC can be seen as the sample was heated past the pre-transition temperature (35 °C) and then through the main transition temperature (41 °C). The K_p for DPPC in the $L_{\beta'}$ phase at 25 °C was 443 vs. 766 for the $P_{\beta'}$ phase at 37 °C and then 866 for the L_α phase at 50 °C. For DOPC which was fluid throughout the temperature range examined, the partition coefficient progressively decreased with the temperature.

Partitioning into binary mixtures of disaturated-PC was also examined. When two disaturated species of PC which have a difference in hydrocarbon chain length of four carbons or greater are mixed at temperatures intermediate between their respective chain melting temperatures, there is phase immiscibility or coexistence of both gel and fluid domains [19]. These lipid dispersions with phase-separated domains represent an interesting model system for investigating partitioning into defect sites which exist at the phase boundaries. To examine how defect sites may influence the association of VM-26 with membranes, we have utilized mixtures combining equimolar amounts of DMPC (T_c = 23 °C) with DSPC (T_c = 55 °C) or DLPC (T_c = −2 °C) with DPPC (T_c =

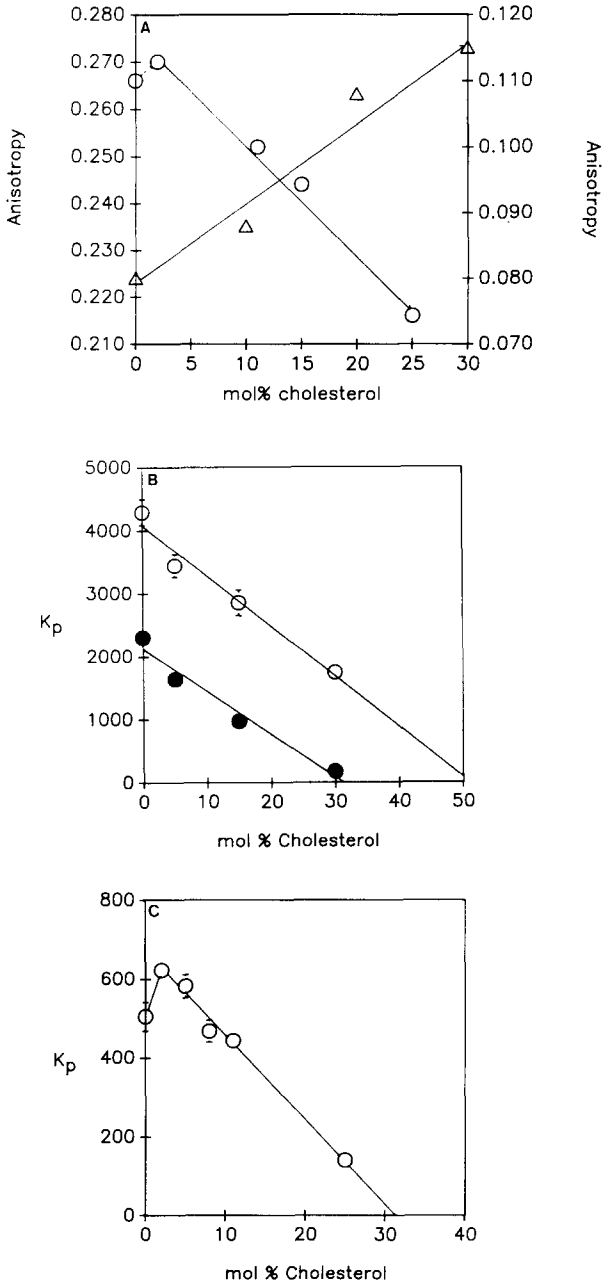

Fig. 4. Effect of cholesterol on partitioning and microviscosity. In panel A the fluorescence anisotropy of DPH in liposomes was examined as a function of cholesterol concentration. The mobility of DPH in fluid DOPC membranes at 25 °C (○, right ordinate) decreased whereas mobility in gel-phase DPPC at 25 °C (△, left ordinate) increased upon addition of cholesterol. In panel B partitioning of VM-26 was measured at 37 °C for DOPC (○) or DMPC (●) as a function of cholesterol concentration. Partitioning into DPPC at 25 °C was examined in panel C.

41° C). The lipids were initially hydrated and mixed at a temperature greater than the highest melting species and then cooled to 37° C for 2–3 h prior to centrifugation. These data are presented in Table II. The shorter chain combination (DLPC/DPPC) accumulated more drug than the longer chain combination which was consistent with the observations from the single component liposomes. More interesting is the observation that the partition coefficients for the mixtures were greater than those of the averages of the individual species. For example, the average K_p for DLPC and DPPC was 1716, yet the K_p for a 1 : 1 mixture of the two lipids was 2081 (Table II). This suggests that the presence of bilayer defects in the form of lipid phase boundaries may facilitate incorporation of the drug into membranes. Cholesterol can also accumulate in these interfacial regions and can effectively anneal the bilayer by eliminating the acyl chain mismatch. Inclusion of 10 mol% cholesterol into DLPC/DPPC (45 : 45) reduced partitioning by 30%. By comparision, partitioning was lowered by 23% in DOPC and by 40% in DPPC (gel phase) in the presence of 10 mol% cholesterol.

Partitioning into mixed-chain single component lipids such as POPC was intermediate between that of the pure disaturated (DPPC) and the diunsaturated (DOPC) forms (Table II). Egg PC accumulated amounts similar to that of POPC which is in agreement with its structural similarity to POPC.

Effect of albumin and serum

The epipodophyllotoxins are known to bind strongly to serum albumin [16] as do most blood borne hydrophobic drugs. To compare the relative binding affinities of VM-26 for lipids and proteins, we have examined the effects of albumin on the partitioning of [^{3}H]VM-26 into high affinity lipids. [^{3}H]VM-26 was incorporated into DOPC as in all other experiments and then incubated with various concentrations of fatty acid-poor bovine serum albumin for one hour prior to centrifugation. As can be seen in Fig. 6 the albumin was able to reduce partitioning in a dose-dependent manner. This line extrapolates to zero partitioning at a BSA concentration of 13 mg/ml which, on a molar basis, is a 67-fold molar excess of protein to drug. Thus, although the albumin was able to extract drug from the liposomes the process was relatively inefficient. However, since the albumin concentration in blood is 44 mg/ml, most of the drug would be expected to transfer to the protein in vivo. The partition coefficient following incubation with 10% calf serum was reduced by almost half to 2190.

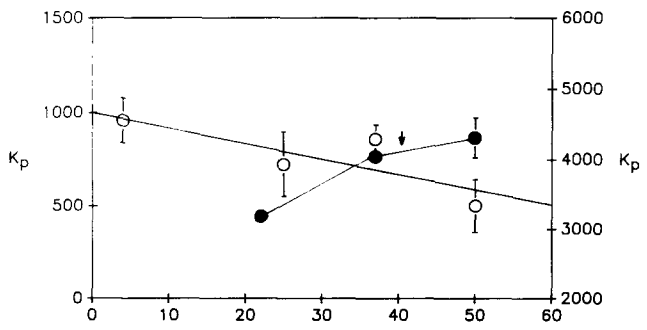

Fig. 5. Partitioning of [^{3}H]VM-26 as a function of temperature. Partitioning into DOPC (○) or DPPC (●) was examined at the indicated temperature. K_p values for DPPC are indicated on the left ordinate and that for DOPC on the right. The transition temperature for DPPC (41° C) is indicated by the arrow.

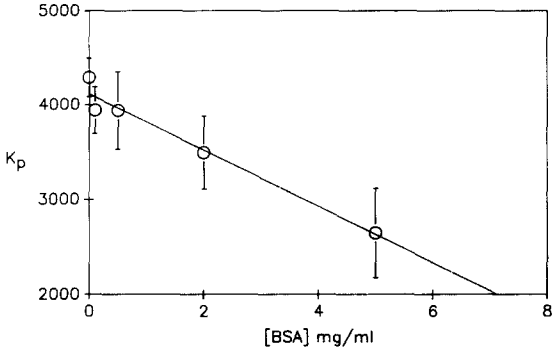

Fig. 6. Effect of albumin on lipid partitioning. Partitioning into DOPC was measured at 37° C as a function of bovine serum albumin concentration. Liposomes were incubated for 3 h before addition of concentrated BSA to the indicated level. Mixtures were then incubated for another hour prior to centrifugation.

This transfer from lipid to protein indicates that the affinity for albumin is even greater than that for lipid. Likewise, the transfer of amphotericin B from liposomes to serum albumin or LDL was found to be essentially complete and also quite rapid [10]. The high affinity of the epipodophyllotoxins for albumin is known from the work of Allen who reported that the epipodophyllotoxins avidly associate with serum albumin with a K_d for VM-26 of 2.7 μM [16]. Thus, the administration of VM-26 incorporated in liposomes may not be a useful device for delivery of these drugs.

Discussion

In this investigation we have attempted to elucidate many of the molecular requirements for the association of VM-26 with bilayer-forming lipids. Our results from partitioning experiments indicate that the epipodophyllotoxins accumulate best in fluid, unsaturated membranes which are free of cholesterol. Moreover, the lipid association of VM-26 was also found to be concentration dependent, lipid species specific and demonstrated a negative temperature coefficient. When compared to other amphipaths, VM-26 has demonstrated partitioning behavior similar to that of many of the well characterized membrane-interactive drugs such as the anesthetics and insecticides [9–13].

The ability of the epipodophyllotoxins to associate with lipids was demonstrated in previous studies in which we examined the effect of these agents on the thermodynamic and interchain motional properties of lipid vesicles [7,8]. The results of these studies indicated that the drugs could intercalate into the bilayer interior where they could perturb the orientation and movement of the acyl chains. Calorimetric studies with DMPC and DPPC suggested that the drug effects were localized in the outer C(2)–C(8) region of the acyl chains [8]. This was substantiated in subsequent experiments in which we found a large ordering effect of VM-26 on doxyl-stearate probes labeled at position 5. The results of the current studies are consistent with drug localization in the near interfacial region.

Although the physical effects of these drugs on membranes appear to be localized to the outer regions of the bilayer core, the findings of the current study indicate that affinity of the drug for lipids can be modulated by structural changes made at almost any location within the bilayer. For example, changing the size or charge of the head group significantly reduced partitioning. Moreover, these head group-mediated effects were not related to changes in acyl chain mobility deep within the bilayer since no differences in DPH polarization was found among these preparations (data not shown). The basis for the preferential association with pure PC vesicles is unknown. One potential explanation for the correlation between phospholipid head group and K_p may be related to relative polarity of the lipids. The charged lipids all generate a polarity profile which is proportional to their net charge at neutral pH. In the case of PE, although it bears the same net charge as PC, the ethanolamine group is thought to be much more hydrophilic than the choline group [20]. Thus, since PE and the negatively charged species are more polar than PC, their inclusion in the PC bilayers may serve to repel the hydrophobic drug from the surface and interfacial region of the bilayer.

Molecular motion in general, and acyl chain mobility in particular, increase with temperature which results in a more fluid membrane environment. Partitioning into DPPC increased sharply when the lipid was heated through its pre-transition temperature and also when heated across its main or chain-melting transition. Thus, the drug apparently favored a more fluid bilayer arrangement. However, if the temperature of fluid liposomes was increased then the interaction between lipid and drug was decreased. We do not believe that this is the result of more rapid efflux of entrapped drug since the drug distributed in the aqueous compartments has been corrected for with the use of [^{3}H]inulin.

A possible explanation for these temperature related effects may be found through thermodynamic considerations. The incorporation of relatively nonpolar molecules like VM-26 across and into interfacial membrane bilayer is thought to be the result of the hydrophobic effect, which, in turn, is driven by entropic factors [21]. The positive entropy of solution which promotes partitioning into hydrophobic domains, is believed to result from the dissolution of an ordered water shell from around the drug. Counterbalancing this effect are enthalpic contributions which enhance the water solubility of hydrophobic solutes. Thus, as the temperature is increased the enthalpy of partition becomes more positive and the free energy of transfer less favorable. These same thermodynamic contributions which promote solvation of drug into lipid could also be responsible for the self-association of these hydrophobic drugs at their CMC. Interestingly, we have found that partitioning of VM-26 from water to octanol is also greater at low temperatures (unpublished). In addition, the steady state levels of VM-26 which can accumulate in Ehrlich cells is greater at 25 °C than at 37 °C (Wright et al., submitted).

Another explanation for the temperature-related effects may be due to the thermally-induced changes in the conformation of the head group. It has recently been suggested that the N^+ end of the phosphocholine dipole of PC which lies parallel to the bilayer surface becomes increasingly submerged in hydrocarbon with increasing temperature, which results in lateral head group repulsion and decreasing surface pressure [20]. Although a reduced surface pressure may be expected to favor partitioning (see below), it may be the altered conformation of the head group which now predominates in terms of drug partitioning. The importance of the head group conformation was also evident when DPPC was cycled through its pre-transition temperature, an event known to involve head group rearrangements.

The reduction of drug partitioning in membranes which were modified by cholesterol could not be attributed directly to membrane fluidity as determined by DPH anisotropy. There is some ambiguity as to the reliability of DPH to accurately reflect acyl chain order. Cholesterol may increase the order parameter for ESR probes in both fluid and gel-phase lipids [24] whereas we and others [25] find that DPH mobility increases in gel-phase lipids titrated with cholesterol but decreases in fluid phase bilayers similarly treated. Thus, any correlation between acyl chain order and drug partitioning remains unresolved since analysis of hydrocarbon mobility within the bilayer may depend upon the probe used to monitor this parameter.

Although the K_p was unrelated to the DPH order parameter, it was inversely proportional to the cholesterol concentration. The two agents, having similar bilayer effects [7] may be competing for common sites within the membrane. The results of this and earlier [7] studies suggest that cholesterol can exclude VM-26. VM-26 probably has little effect on cholesterol partitioning since its effect on bilayer properties is not

altered upon addition of the drug [7]. The ability of cholesterol to reduce the bilayer concentration of VM-26 may explain the attenuated ordering effects of the drug as the cholesterol concentration is increased [7]. The effects of cholesterol on K_p may also be related to its influence on bilayer surface density as it has recently been shown that cholesterol increases the surface density of either fluid or gel phase lipids [22]. This condensing effect of cholesterol at the interfacial region of the bilayer can exclude solutes such as benzene for which the partition coefficient was found to be inversely proportional to the surface density when modified by cholesterol [22]. The ability of cholesterol to exclude VM-26 from either fluid or gel phase saturated PC and also unsaturated PC in the fluid phase indicates that a similar mechanism which is independent of fluidity gradients may apply to our system.

Although the mechanism of action of many amphipathic agents are thought to be mediated through their interaction with membranes, we cannot say at this time if this pertains to the epipodophyllotoxins as well. However, even if the lipophilicity of these anticancer agents does not contribute directly to their cytotoxicity, it at least facilitates their rapid entry into cells since their permeability is dependent upon their ability to enter into and cross the plasma membrane. In addition, since they are lipophilic, these drugs can be retained and become concentrated within the lipid domains of membranes where they may influence the rate and extent of uptake of other anticancer drugs [4,5].

One of the common manifestations of cellular resistance to these agents is reduced uptake into the cell. Even though the phenomena of multiple drug resistance (MDR) has received much attention in recent years, the molecular basis of the process by which cells exhibit cross-resistance to a variety of anticancer agents remains unresolved. Beck and co-workers [18] have recently suggested that one common characteristic of drug which can circumvent MDR and increase the cytotoxicity of VM-26 and vinca alkaloids is their lipid solubility. Although the drugs which could enhance cytotoxicity were reported to be lipid soluble, this parameter was not directly measured but was inferred from octanol/water partition coefficients. As it is becoming increasingly evident in the literature, octanol/water coefficients are frequently not an accurate estimate of lipid solubility. This is because lipid dispersions are interfacial, anisotropic compartments which can be much more discriminating than bulk solvent. Our present findings indicate that VM-26 is in fact soluble in lipids as well as octanol. Moreover, depending upon the lipid, we have found partitioning of VM-26 into lipid to be 5–13-fold greater than partitioning into octanol. We believe this degree of lipophilicity may contribute to its concentrative accumulation within cells. One of the frequent findings in many MDR cells is that they accumulate low levels of drug relative to their sensitivity counterparts. Knowledge of the relative lipophilicity of the epipodophyllotoxins may contribute to our understanding of one type of multiple drug resistance.

The ability of drugs to tightly associated with lipids may allow for therapeutic alternatives for the administration of these agents. For example, a liposome-base formulation of the hydrophobic polyene antibiotic amphotericin B has been found to have several advantages as compared to the free drug. This type of drug carrier confers a selectivity of delivery for amphotericin B while reducing the dose-limiting nephrotoxicity [26]. The liposome composition has been found to be very important for maintaining these properties. The current studies indicate that while liposomal formulations of VP-16 or VM-26 may eliminate the need for emulsifiers, once administered the drug will eventually be transferred to serum proteins. Therefore, the utility of using the hydrophobic domains of liposomes as carriers for the epipodophyllotoxins does not look promising. An alternative is to entrap these drugs at high concentrations within the aqueous compartments of liposomes composed of lipid species which have a poor affinity for the drug which should facilitate retention. For example, entrapment in DPPE/cholesterol mixtures may serve as a slow-release carrier for these agents.

In summary, we have shown VM-26 to be very soluble in lipid membranes but that this solubility is strictly dependent upon the physical state of the bilayer. The drug shows a strong preference for pure PC membranes in the fluid state and inclusion of any additional components, other than VP-16, reduces its membrane association. Thus, the simple presence of a non-polar lipid domain is not in itself sufficient to attract the drug but it must also conform to strict structural requirements needed for drug accumulation and retention.

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