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Membrane ordering effects of the anticancer agent VM-26

Stephen E. Wright and J. Courtland White

Department of Biochemistry, Bowman Gray School of Medicine, Winston-Salem, NC 27103 (U.S.A.)

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The effect of the anticancer agent VM-26 on acyl chain order of cellular and model membranes was examined by electron spin resonance techniques. The order parameter for the paramagnetic probe 5-doxyl stearate was increased when VM-26 was incorporated into the bilayer of fluid-phase dimyristoylphosphatidylcholine (DMPC) or gel-phase dipalmitoylphosphatidylcholine (DPPC) liposomes at concentrations up to 4.8 mol%. The ordering effect of VM-26 in DMPC was greater than that of cholesterol on an equimolar basis. The less cytotoxic congener of VM-26, VP-16, was only one-third as active as VM-26 in its ordering effects on DMPC. Higher order parameters for 5-doxyl stearate were also noted in asolectin liposomes, Ehrlich ascites tumor cells, and CCRF-CEM cells treated with VM-26. We conclude that VM-26 has significant membrane associated activity in addition to its previously recognized nuclear effects.

Introduction

The epipodophyllotoxins are amphipathic anticancer agents which have been developed within the last decade. VM-26 (4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside, or teniposide) is a semisynthetic podophyllotoxin derivative that has been shown to be active against a variety of solid tumors and leukemias [1]. Although its effectiveness in killing cells has been ascribed primarily to its effects on nuclear enzymes [2], many membrane associated actions have also been observed [3–6]. Facilitated transport of nucleosides and therapeutic nucleoside analogs is inhibited by pharmacologically relevant concentrations of the epipodophyllotoxins [3–5]. The separate carriers that mediate the influx and efflux of the antifolate methotrexate are also inhibited by the epipodophyllotoxins [6].

In spite of the many membrane-related activities of the epipodophyllotoxins, there has been no systematic study of the possible mechanism(s) responsible for these effects. The inhibition by VM-26 of dissimilar membrane-associated processes suggests that the effects may be due to nonspecific membrane perturbations. The amphipathic nature of VM-26 would allow for associations either with membrane lipids or with hydrophobic domains on membrane proteins. VM-26 has recently been shown to interact with model membrane lipids. Evidence for this interaction was obtained by differential scanning calorimetry studies which indicated that concentrations as low as 1 mol% VM-26 changed the melting profile of phosphatidylcholine liposomes [7].

The plasma membrane is increasingly viewed as a mediator of cytotoxicity for many anti-neoplastic agents which were previously thought to act

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid; TLC, thin-layer chromatography.

Correspondence: Dr. J.C. White, Department of Biochemistry, Bowman Gray School of Medicine, Winston-Salem, NC 27103, U.S.A.

only through their interactions with nuclear elements [8]. As an initial site of contact, the plasma membrane represents both the first barrier as well as the first potential target for the incoming drug. Membrane interactive properties of these compounds could therefore be important determinants of their cellular effects. Although many antineoplastic agents have been shown to alter membrane order [8], the significance of this effect with regard to their therapeutic action remains unclear. However, since transformed cells in general have altered membrane fluidity [9], modulation of this parameter may be expected to contribute to the action of antineoplastic drugs. The current study was undertaken to further evaluate the effects of VM-26 on general membrane properties such as acyl chain order.

Materials and Methods

VM-26 was obtained from the Pharmaceutical Research and Development Division of Bristol Myers Co., Syracuse, NY. The electron spin resonance (ESR) labels were the stearic acid probes 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl [I(12,3)] (5-doxyl stearate) and 2-(10-carboxydecyl)-4,4-dimethyl-2-pentyl-3-oxazolidinyloxyl [I(5,10)] (12-doxyl stearate) which were obtained from Molecular Probes, Junction City, OR. Spin labels were brought up in ethanol to 5 mM and stored at −20°C.

Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma Chemical Co., St. Louis, MO. The lipids migrated as a single spot by TLC and were used without additional purification. Asolectin was a gift from Dr. E. Racker, Cornell University. All other compounds were of reagent grade or better and were obtained from Sigma.

Ehrlich ascites tumor cells were grown in male CF-1 mice (Charles River Breeding Laboratories, North Wilmington, MA) as previously described [5]. Tumor transplantation was by biweekly intraperitoneal inoculation of 0.2 ml of undiluted ascites fluid. Cells were harvested 7 to 9 days after injection. The cells were washed twice in cold phosphate-buffered saline (140 mM NaCl/10 mM K_2HPO_4:KH_2PO_4/pH 7.4, PBS), and contaminating erythrocytes were removed by hypotonic lysis [5]. Plateau phase CCRF/CEM cells that had been cultured in Fishers media + 10% horse serum were a gift of Dr. D. Fernandes, Bowman Gray School of Medicine. Cells were resuspended in phosphate-buffered saline at a cell density of $3 \cdot 10^7$ cells/ml for all experiments. In the presence of 50 μM VM-26 previous estimates [7] indicate that the molar ratio of VM-26 to phospholipid is approximately 2%.

Liposome dispersions were formed by first depositing chloroform/methanol (2:1, v/v) solutions of the given lipid into 12 × 75 mm tubes and then evaporating the solvent under a stream of nitrogen or argon. Residual solvent was removed under vacuum overnight. Lipid films were hydrated with 10 mM Hepes buffer (pH 7.4) which had been warmed to a temperature approximately 10 K above the phase transition of the given lipid. The lipids were dispersed by vortex mixing and maintained above their phase transition temperatures for at least 1 h prior to ESR analysis. Liposomes generated by this method are known to form multilamellar structures [10]. Drug containing liposomes were formed as described with the drug co-deposited as a chloroform/methanol solution along with the lipid in the same solvent.

The spin labels used in this study partition into cellular membranes and are oriented with the carboxyl group adjacent to the phospholipid head group and the acyl chain oriented perpendicular to the bilayer normal [11]. The motion of the 5- and 12-doxyl stearate probes report on the fluidity near the surface and near the interior of the bilayer, respectively. For ESR experiments with liposomes, 5 nmol of label was added to 5 μmol of lipid to give a probe to lipid ratio of 0.1%. For studies with intact cells, VM-26 (in DMSO) was added and the cells were incubated for 10 to 20 min at 25°C. The solvent concentration was kept below 0.2% (v/v) and was added to the drug-free cells as a control. The fatty acid spin labels were then added to the cell suspension to a final concentration of 10 μM. The samples (500 μl) were placed in a quartz flat cell for the ESR experiments.

ESR spectra were recorded after a 5 min period for temperature equilibration with a Bruker ER 200 D ESR spectrophotometer equipped with a Bruker ER 4111 VT variable temperature acces-

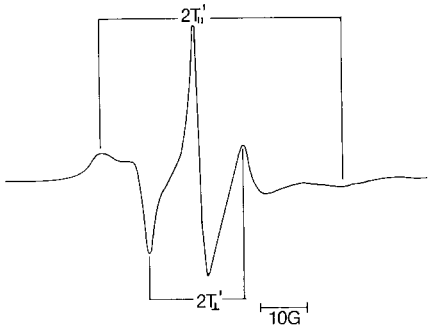

Fig. 1. ESR spectrum of 5-doxyl stearate in DMPC at 26°C. Order parameters were determined from the indicated extrema as described in the text.

sory. All experiments were carried out at 299 K (26°C) which is above the phase transition temperature for DMPC (23°C) and below that for DPPC (41°C). Data were collected and evaluated on an Aspect 2000 minicomputer interfaced with the spectrophotometer. Spectra were obtained over a 100-gauss (G) range centered at 3420 G, with 21.2 mW microwave power, 1 G modulation amplitude, 20 ms time constant, and time-averaged spectra of 20 to 30 scans with scan times of 10 s each were collected. A representitive spectrum of 5-doxyl stearate in fluid-phase DMPC is shown in Fig. 1. The order parameters (S) were calculated using the indicated hyperfine splittings according to the method of Hubbell and McConnell [11] with correction of the $T_{\perp}$ value by the method of Gaffney [12].

Results

The ESR spectra obtained were indicative of rapid anisotropic motion resulting from rotational and segmental movement within the bilayer. The data are presented as order parameters which are related to the time-averaged angle that the nitroxide axis of the probe makes with the bilayer surface and are thus a measure of the flexibility of the probe. The order parameter is assigned a value of 0 to 1, with higher values representing more ordered or less flexible environments. Any factors which alter probe motion will result in a change in the order parameter for the receptor molecule.

Addition of teniposide to any of the liposome systems resulted in increased order parameters for

TABLE I

EFFECT OF VM-26 ON THE ORDER PARAMETERS FOR 5-DOXYLSTEARATE IN DMPC, DPPC, OR ASOLECTIN LIPOSOMES AT 26°C

Samples contained 10 mM lipid, 10 μM spin label and the indicated concentration of VM-26 or VP-16. Spectra were collected at 26°C which is above the T_m for DMPC (23°C) and below that for DPPC (41°C).

Lipid	Addition	Concn. (mol%)	Order parameter $S \pm$ S.E. (n)
DMPC	None	–	0.588 ± 0.001 (14)
	VM-26	1.0	0.592 ± 0.001 (3)
		4.8	0.618 ± 0.002 (11)
		9.1	0.619 ± 0.001 (3)
	VP-16	4.8	0.599 ± 0.001 (3)
DPPC	None	–	0.709 ± 0.015 (4)
	VM-26	4.8	0.728 ± 0.003 (4)
Asolectin	None	–	0.569 ± 0.003 (3)
	VM-26	4.8	0.579 ± 0.004 (3)

5-doxyl stearate. When VM-26 was incorporated into fluid DMPC dispersions at concentrations of 1 to 4.8 mol%, there was an increase of 0.6 to 5.1% in the order parameter (Table I). Although at no point was there a cross-over to a disordering effect as has been described for other membrane-active agents [13], there was a plateau for maximal drug-induced ordering. Since no additional change in the order parameter was produced by increasing the concentration of VM-26 above 4.8 mol%, an optimal drug/lipid ratio which results in maximal ordering effects apparently exists. Drug concentrations up to 5 mol% were chosen because they were believed to be close to those obtainable in vitro and in vivo [7].

VP-16 (etoposide) is a more hydrophilic congener of VM-26, having a methyl substitution in place of the thiophene. Compared with VM-26, this agent is less cytotoxic [2] and is less active as an inhibitor of membrane transport [5,6]. VP-16 was found to have only one-third the effect of VM-26 at ordering the DMPC bilayer (Table I).

Spectra were collected on DPPC at 26°C (T_m = 41°C) to determine if VM-26 had the same effects on lipids in the gel phase as it did on those in the liquid-crystalline phase. The data in Table I indicate that VM-26 hindered the mobility of 5-doxyl stearate in gel-phase DPPC, although not as

extensively as that noted for fluid-phase DMPC. Although addition of 4.8 mol% VM-26 has been shown to lower and broaden the transition temperature for DPPC [7], at 26°C the lipid is in the gel phase in both untreated or drug-treated DPPC liposomes.

The actions of VM-26 on membrane order were also examined in asolectin, a mixed lipid system. This heterogeneous lipid consists largely of mixed chain phosphatidylcholine and phosphatidylethanolamine and therefore provides a more fluid structure than the fully saturated DMPC. An examination of the order parameters for the untreated liposomes indicated that the mixed lipid membrane was, in fact, less ordered. Addition of 4.8 mol% VM-26 to these liposomes increased the order parameter for 5-doxyl stearate by 1.8%. This is only 30% of the effect that is seen when equivalent concentrations of drug are incorporated into DMPC and probably reflects differences in the basal order of the two lipid systems.

Cholesterol is known to be a potent modulator of membrane structure. The predominant effect of cholesterol incorporation is a large increase in membrane order [14]. We have compared the membrane ordering effects of VM-26 and cholesterol on DMPC by ESR. Cholesterol alone induced dose-dependent ordering of the lipid as reported by 5-doxyl stearate (Fig. 2). When compared at equimolar concentrations (4.8 mol%), VM-26 was more potent than cholesterol in ordering ($S = 0.618$ vs. 0.604). The leveling off of the ordering effect by cholesterol at approx. 33 mol% is similar to that noted for VM-26 at 4.8 mol%. This break-point for cholesterol had also been reported in fluid-phase DPPC [15].

Cholesterol has been shown to modulate the membrane effects of many membrane-acting drugs [13]. We have investigated the combined effects of cholesterol and VM-26 at VM-26 concentrations which produced maximal effects alone. Addition of 4.8 mol% VM-26 to DMPC liposomes containing variable cholesterol concentrations resulted in consistently higher order parameters than for cholesterol alone (Fig. 2). Even at the highest cholesterol concentrations studied (50 mol%) there was an additional ordering effect of VM-26. The cumulative effects of the two compounds were apparently sub-additive, especially at higher cholesterol concentrations where the ordering effect of VM-26 was apparently attenuated by cholesterol.

The effect of VM-26 on the membrane order of intact Ehrlich ascites tumor cells was examined with both 5-doxyl stearate and 12-doxyl stearate probes (Table II). When the cells were incubated with 50 μM VM-26 the mobility of both probes was reduced, suggesting an increase in plasma membrane order. The order parameter for 5-doxyl stearate was increased by 2.9% whereas that for 12-doxyl stearate was increased by 4.1%. The concentration of VM-26 which was used for these studies was a concentration which has been shown to inhibit carrier-mediated transport systems [5,6].

The membrane-ordering activity of VM-26 was not limited to the Ehrlich cell. VM-26 also produced ordering effects in the CCRF/CEM human leukemia cell line (Table II); however, the effect was less than that observed in the Ehrlich cell. The

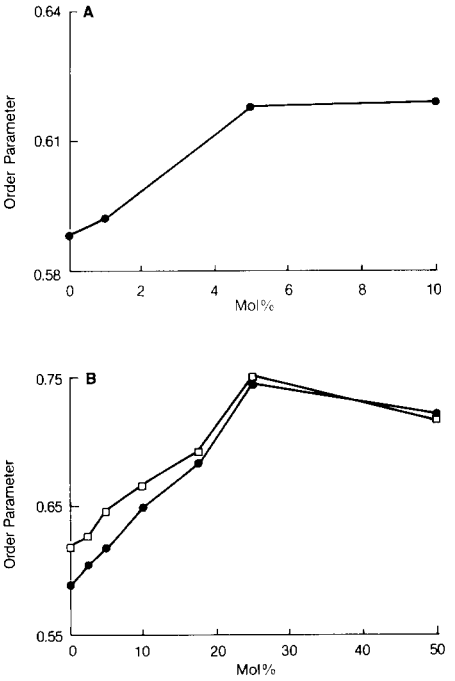

Fig. 2. Effect of VM-26 and cholesterol on order parameters for 5-doxyl stearate in DMPC at 26°C. (A) Effect of VM-26 alone on order parameters. (B) Effect of cholesterol (●) or cholesterol plus 4.8 mol% VM-26 (□) on order parameters. Standard error bars are contained within the symbols.

TABLE II

EFFECTS OF VM-26 ON ORDER PARAMETERS IN EHRLICH OR CCRF/CEM CELLS

Label: 5-SASL, 5-doxyl stearate spin label; 12-SASL, 12-doxyl stearate spin label.

Cell type	Addition	Concn. (μM)	Label	Order parameter $S \pm$ S.E. (n)
Ehrlich	None	–	5-SASL	0.626 ± 0.004 (7)
	VM-26	50	5-SASL	0.644 ± 0.005 (7)
	None	–	12-SASL	0.171 ± 0.002 (7)
	VM-26	50	12-SASL	0.178 ± 0.002 (7)
CCRF/CEM	None	–	5-SASL	0.569 ± 0.004 (3)
	VM-26	50	5-SASL	0.579 ± 0.004 (3)

order parameter for 5-doxyl stearate was increased by only 1.8% by 50 μM VM-26. This effect was anticipated since the order parameter in the untreated CCRF/CEM cells was less than that for the Ehrlich cells. This data with intact cells is consistent with the above data with DMPC and asolectin and indicates that VM-26 has its greatest ordering effect in relatively rigid bilayers.

Discussion

This examination of the interaction of the anticancer agent VM-26 with model and cellular membranes has shown that this amphipathic agent consistently orders membranes. This finding has confirmed the previous observation that VM-26 interacts with membrane lipids [7] and extends the view that this agent has significant membrane perturbing properties in addition to its well known nuclear effects [2].

It is unlikely that the effects of VM-26 on the mobility of the spin label were the result of direct drug–probe interactions. The drug/spin label ratio ranged from 10/1 to 100/1 over the range of VM-26 concentrations examined. If there was a distinct interaction between the two amphipaths, it would probably involve intermolecular hydrogen bonding between the oxy radical and the pendulum phenolic group. Thus, there should not be any additional slowing of the probe motion at ratios greater than one (corresponding to 0.1 mol% VM-26). There was, however, progressive ordering at ratios of 10/1 to 50/1 (Fig. 2). We have also examined the effect of VM-26 on membrane order by another technique using a completely different reporter molecule. Fluorescence polarization studies with diphenylhexatriene as the probe confirmed the VM-26 ordered DMPC bilayers (data not shown).

Further, we do not believe that the observed effects on membrane order are due to degradation products of VM-26 for the following reasons. The ordering effect of VM-26 was observed in liposomes in which there were no hydrolytic enzymes present. In addition, the comparative potencies of VM-26 and VP-16 suggests that the molecule is remaining intact. If ordering was due to just a dissociated ring (i.e. the aglycone) then the potency of the two should be equal. Lee and Roberts [38] did not observe degradation products when several different L1210 sublines were incubated with VM-26.

The ability of cholesterol to order membrane is well documented. ESR techniques have been widely used to show that 1–50 mol% cholesterol orders liposomes of egg PC [15,16] and several disaturated species of PC in either the fluid phase [15,17] or the gel phase [17,18]. Cholesterol-induced ordering has also been shown by Raman spectroscopy in which it was shown that, in fluid-phase DMPC, there was an increase in the number of *trans* C-C bonds [19].

The ordering ability of VM-26 is especially striking when compared to cholesterol. At equimolar concentrations (4.8 mol%), order parameters for VM-26 treated liposomes were increased by 5.1% as compared with a 2.7% increase for cholesterol. Cholesterol is believed to decrease membrane fluidity through the interaction of the rigid steroid nucleus with the adjacent fatty acid chains, inhibiting the formation of kinks resulting from a decrease in the number of gauche conformations within the acyl chain. Hydrogen bonding between the 3β-hydroxy group of cholesterol and the carbonyl oxygen of the phospholipid is critical for condensing effects to appear [20]. It is of interest that VM-26 has 3 hydroxyl groups available for hydrogen bonding with the phospholipids. Hydrogen bonding of VM-26 with more than one phospholipid could lead to potential cross linking between phospholipids which would decrease their mobility and thus account for the increased order

seen in the presence of the drug. The quantitatively greater ordering effect of VM-26 over cholesterol in DMPC bilayers is consistent with this concept.

The leveling off of the ordering effects by VM-26 at about 5 mol% and cholesterol at 33 mol% indicate that both agents may be interacting with the bilayer by a common mechanism but at apparently different stoichiometries. These observations of an ordering maxima are not without precedent. Kusumi et al. [15] recently observed that for cholesterol-enriched DPPC in the range of 0–50 mol% cholesterol the mobility of 5-doxyl stearate decreased monotonically with addition of cholesterol up to 32 mol%, at which point there was a progressive decrease in order upon further addition. Breaks at 33 mol% cholesterol have also been previously detected in fluid-phase DLPC [15], DSPC [15], and confirmed for DPPC [17]. For DMPC the break has been reported to appear at approx. 25 mol% cholesterol [15]. Both X-ray diffraction [21] and calorimetric studies [22] have shown that there is an abrupt change in the physical properties of the bilayer at 33 mol% cholesterol. Moreover, for cholesterol/lipid mixtures there appear to be phase boundaries at 20 and 33 mol% cholesterol as determined by NMR [23] and ESR [18] techniques.

The ability of VM-26 to order membranes regardless of their composition is evident by the data from asolectin or cultured cells. Following addition of VM-26, the order parameters for 5-doxyl stearate were increased 1.8%, 1.8%, and 2.9% for asolectin liposomes CCRF/CEM cells, and Ehrlich cells, respectively. Since this is much less than the effects noted with DMPC, it can be concluded that the ordering ability of VM-26 decreases with increasing degree of unsaturation. Unsaturation of the acyl chains greatly reduces the ordering effect of cholesterol at both the C-5 and C-16 positions of the acyl chains [15].

Earlier studies of VM-26-lipid interactions suggested that the drug was residing in the C-2 to C-8 region of the bilayer [7]. The profound effects of VM-26 on the spin label monitoring approximately the C-5 region would support this conclusion. However, the order parameter for 12-doxyl stearate was increased to a greater extent than that for 5-doxyl stearate in Ehrlich cells (Table II). This indicates that the drug is either located deeper within the bilayer than expected or that the ordering effects are somehow amplified down the acyl chains. Cholesterol, although believed to reside near the surface of the membrane, also has a greater ordering effect on 12-doxyl stearate than on 5-doxyl stearate [16,24]. This suggests that, in general, the more fluid regions deep in the membrane are more sensitive to membrane perturbants than are the more rigid surface regions [24].

Membrane fluidity is known to be important in regulating membrane activity and biological function [25]. Small changes in the fluid state of the membrane can result in large changes in the activity of membrane-bound proteins [26,27]. For example, changing the order parameter by only 1–2% has been shown to change the activity of $(Na^+ + K^+)$-ATPase by up to 250% [27]. In addition, membrane transport is thought to be dependent on the immediate lipid environment of the integral proteins. Furthermore, it has been shown that a decrease in membrane fluidity results in an increase in exposure of protein reactive groups to the aqueous polar environment [28,29]. Changes in lipid fluidity can thus expose or conceivably mask membrane proteins such as transport carriers [30].

Many different types of drugs are known to modulate membrane fluidity [31]. Some of the antineoplastic agents which alter fluidity by increasing the order of the membrane include the nitrogen mustards [32], chlorambucil [32], and adriamycin [8,33]. VM-26 may now be added to this list of agents which have no common mechanism of action other than that of a membrane perturbant. In addition, VM-26, chlorambucil, adriamycin, and most of the nitrogen mustard derivatives have a cyclic ring moiety in common. It has been pointed out that most of the anesthetic-like compounds which order phospholipid bilayers also contain chemical ring structures, suggesting that they may act in a manner similar to cholesterol [13]. If these agents are acting in a manner analogous to cholesterol then a combination of drug and cholesterol should have additive effects at least through the linear region of the dose-response curve up to 50 mol% cholesterol [13]. We see from the data in Fig. 2 that there is a subadditive effect on membrane

order for 4.8 mol% VM-26 plus 4.8 to 33.3 mol% cholesterol. Moreover, we did not detect any cross-over from ordering to disordering effects of VM-26 in the presence of high concentrations of cholesterol as has been reported for drugs producing similar increases in order parameters [13]. The effect of VM-26 at 10% was diminished from that observed at 5%, suggesting that very high drug concentrations may eventually produce a disordering effect. Such a conversion from ordering to disordering at higher concentrations has been reported for some of the anesthetics [34,35]. The lesser effect of VP-16 as compared to VM-26 in membrane ordering is most probably related to its lower partitioning into the lipid phase. The oil/water partition coefficients for VM-26 and VP-16 and 7.2 and 2.7, respectively [36]. Anesthetics have been shown to have variable ordering potencies when compared at total concentrations but when compared at similar membrane concentrations they were equally effective [37]. This suggests that if equimolar concentrations of VM-26 and VP-16 were incorporated into the bilayer they may be equipotent in their ordering ability.

The membrane perturbing actions of VM-26 may be important for cancer chemotherapy. Since almost all agents entering the cell must do so either by diffusion or carrier-mediated mechanisms, membrane-related effects of VM-26 may contribute to the cytotoxicity of coadministered agents as well as its own. The flux of cytosine arabinose [5] and methotrexate [6] have already been shown to be inhibited by pharmacologically relevant concentrations of epipodophyllotoxins.

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