Also, it could be demonstrated that compounds which are structurally related to catecholamines, such pyrocatechol and 3,4-dihydroxymandelic acid, but devoid of β-adrenergic activity, did not compete for the binding sites. At 1 mM concentration, these compounds inhibited the binding of (−)-[^{3}H]DHA to mammary membranes by less than 6.6 and 17.2% respectively.

Finally, a functional response to mammary β-adrenergic receptor triggering was verified by measuring the increase of cyclic AMP production after isoproterenol stimulation of adenylate cyclase in lactating mammary gland membranes (Fig. 3). These experiments confirmed the inhibition of lactose production by epinephrine, isoproterenol and dibutyryl cyclic AMP, reported by Loizzi *et al.* [1].

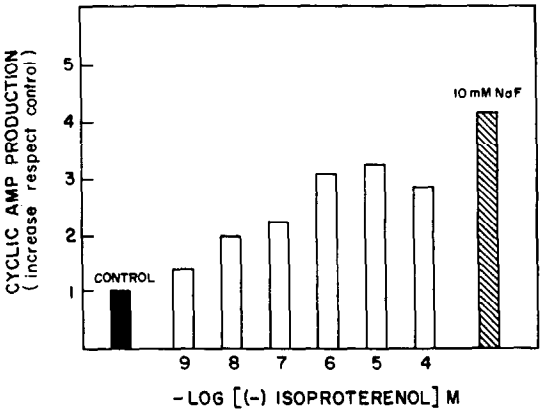

Fig. 3. Increase in cyclic AMP production after isoproterenol stimulation of adenylate cyclase in lactating mammary gland membranes (17 days). Mammary gland membranes were incubated in the presence of an ATP regenerator system and increasing concentrations of (−)-isoproterenol (ranging from 10^{-4} to 10^{-9} M) for 15 min at 37°. The data show the mean from two experiments with triplicate incubations and duplicate determinations of cAMP.

It may be concluded that the rat mammary gland has β-adrenergic receptors which are functionally operative and which have specificity and affinity characteristics similar to those described for other cells and tissues. Studies on the changes in the characteristics of the β-adrenergic receptors and of the number and distribution of the α-adrenergic receptors in relation to the lactation cycle are now in progress in order to establish their role in the process of lactogenesis.

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Microsomal interactions and inhibition of lipid peroxidation by etoposide (VP-16, 213): Implications for mode of action*

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The etoposide VP-16, 213 (NSC 141540, Fig. 1), a semi-synthetic podophyllotoxin derivative, is a promising anti-tumour agent for the treatment of small cell lung carcinoma [1, 2] and currently is undergoing extensive clinical evaluation. While the parent podophyllotoxin (PDP, Fig. 1) binds to microtubulin protein and arrests cells in metaphase [3], VP-16 has been shown to induce single-stranded breaks in DNA of HeLa cells [4] and to inhibit incorporation of nucleic acid precursors [5]. Recent studies by Wozniak and

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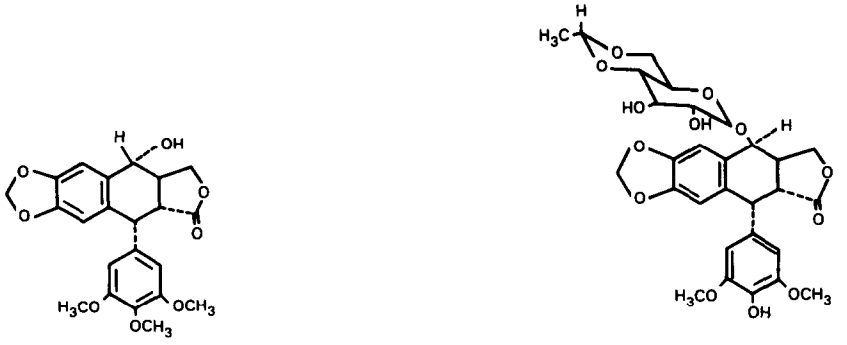

Fig. 1. Structures of VP-16-213 and podophyllotoxin (PDP).

Ross [6] have shown that VP-16 induces single- and double-stranded DNA breaks in L1210 cells and isolated nuclei and have suggested that DNA damage may be responsible for VP-16 cytotoxicity.

VP-16 is also used in combination with other chemotherapeutic agents including adriamycin, *cis*-platinum and cyclophosphamide, resulting in increased therapeutic index and decreased toxicity [7]. The anthracycline antitumor drugs adriamycin and daunomycin undergo reduction–oxidation reactions when enzymatically activated and produce oxygen-derived reactive species ($\dot{O}_2^-$ $\dot{O}H$ and H_2O_2) which initiate lipid peroxidation [8, 9]. Lipid peroxidation has been implicated in anthracycline-induced cardiotoxicity [10, 11]. In a preliminary communication [12], we have shown that VP-16 inhibits daunomycin-induced microsomal lipid peroxidation and forms an oxygen-centered free radical during peroxidative activation. We have extended these observations and have further characterized the inhibition of lipid peroxidation by VP-16 and its parent compound. It also appears that VP-16 undergoes O-demethylation during activation with rat liver microsomes in the presence of NADPH. These results may have implications for the mode of action of VP-16 and its use in combination chemotherapy.

Materials and methods

VP-16 (NSC 141540) and daunomycin (NSC 82510) were obtained from the Drug Development Branch, National Cancer Institute, NIH, Bethesda, MD. Podophyllotoxin (PDP) was obtained from the Aldrich Chemical Co., Milwaukee, WI. Thiobarbituric acid (TBA), NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, phenobarbital sodium salt (PB), xanthine oxidase and polypropylene glycol (mol. wt ~ 200) were obtained from the Sigma Chemical Co., St. Louis, MO.

Mouse hepatic microsomes and phenobarbital-induced hepatic microsomes (PB dose, 80 mg/kg, i.p. × 3 daily) were prepared from CDF_1 male mice according to a previously published method [13]. Food and water were provided *ad lib*. Microsomal protein was determined by the method of Sutherland *et al.* [14] with bovine serum albumin as the standard. Peroxidation of the microsomal lipids was measured by thiobarbituric acid assay [9]. VP-16 and PDP were dissolved in dimethyl sulfoxide (DMSO) and added to the microsomal incubations. Controls contained an equivalent amount of DMSO (final concentration = 0.5%). DMSO, at this concentration, had no effect on the lipid peroxidation. NADPH oxidation was measured by monitoring the loss of absorbance of NADPH (0.1 mM) at 340 nm [15] using an Aminco DW-2a spectrophotometer in the split-beam mode. The reference cuvet was divided into two compartments; double concentrations of microsomal proteins and drugs were mixed in one compartment, and NADPH (0.2 mM) was included in the other. This arrangement compensated for any interference by the drugs in the measurement of NADPH oxidation [15]. O-Demethylation studies were carried out by determining formaldehyde formation during microsomal-NADPH incubations of VP-16 according to the method of Nash [16]. Aminopyrine and *p*-nitroanisole were used as positive controls in the assay.

Oxygen uptake was determined with a Clark electrode in a Water-Jacketed glass vessel filled with 150 mM KCl–50 mM Tris buffer (pH 7.4) at 37°. The drugs (VP-16; 100 μM) and daunomycin (100 μM) were incubated with the microsomes (1 mg/ml) for 2 min; the reaction was initiated by adding NADPH (1 mM), and the rate was taken as the initial slope.

Results and discussion

Our preliminary report [12] showed that VP-16 forms a stable oxygen-centered free radical intermediate during incubations with HRP and H_2O_2. PDP, under the identical conditions, did not produce any detectable ESR signal, indicating that the radical intermediate formation required a free hydroxyl group in the 4′-position. The significance of the free hydroxyl group in the biological properties of VP-16 is further illustrated when their effects on microsomal lipid peroxidation are examined. The anthracycline antitumor drugs adriamycin and daunomycin produce reactive oxygen species ($\dot{O}_2^-$, $\dot{O}H$, H_2O_2), which induce lipid peroxidation [8, 9]. Incubation of daunomycin with microsomes in the presence of an NADPH-generating system and oxygen induced the formation of thiobarbituric acid reactive malonaldehyde (MDA) which increased with time. However, inclusion of 50 μM VP-16 in the incubation mixtures inhibited both the basal and daunomycin-promoted lipid peroxidation (Fig. 2A). In contrast, PDP, under identical conditions, had a much lesser effect on this peroxidation (Fig. 2A), suggesting that the presence of 4′-OH plays an important role in this antioxidant effect of VP-16. The inhibition of the lipid peroxidation was also dose dependent (Fig. 2B), and as little as 25 μM VP-16 inhibited daunomycin-promoted MDA formation by 50%.

Since VP-16 was a potent inhibitor of endogenous and daunomycin-promoted microsomal lipid peroxidation, we investigated the effects of VP-16 on NADPH oxidation in the presence of microsomes. VP-16, at a concentration (50 μM) where the daunomycin-promoted lipid peroxidation is inhibited, did not alter the rate of NADPH oxidation (Table 1). However, it increased daunomycin-enhanced NADPH oxidation (Table 1). These observations suggest that VP-16 does not inhibit the transfer of electrons from NADPH to the flavin moiety of cytochrome P-450

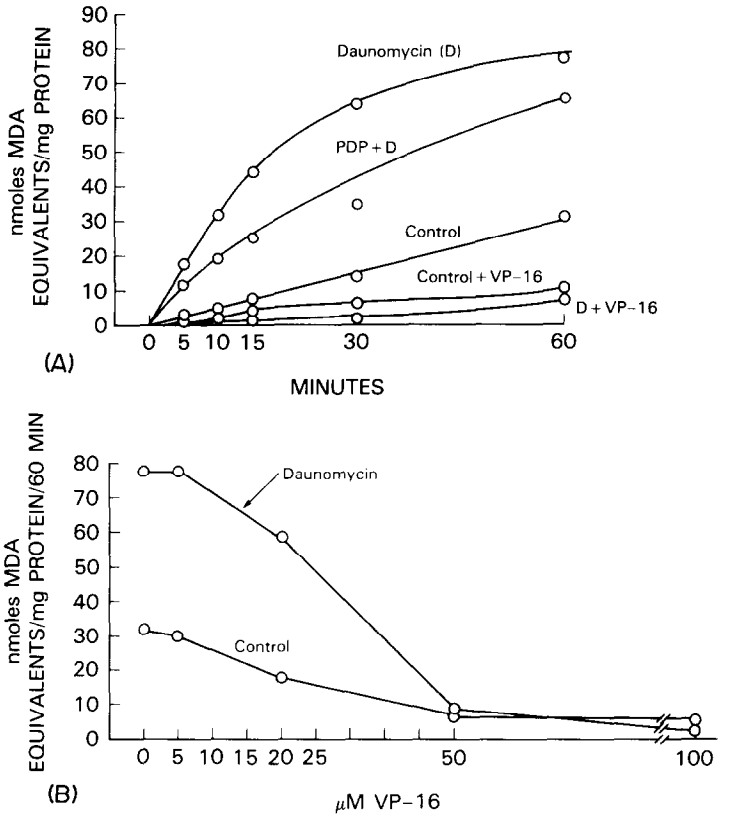

Fig. 2. (A) Time-course for mouse liver microsomal lipid peroxidation in the presence of daunomycin (100 μM) and the effects of VP-16 (50 μM) and PDP (50 μM) on the peroxidation. (B) Concentration-dependent effects of VP-16 on control and daunomycin (100 μM) induced microsomal lipid peroxidation. The microsomes were incubated with various concentrations of VP-16 in the absence and the presence of daunomycin for 60 min under oxygen atmosphere. Each point represents the mean ± S.D. of three experiments.

Table 1. Effects of daunomycin and VP-16 on NADPH oxidation and oxygen uptake during mouse liver microsomal incubation*

Drugs	NADPH oxidation (nmoles/mg protein/min)	O_2 uptake (nmoles/mg protein/min)
None	17.7 ± 1.3	11.7 ± 1.1
VP-16 (50 μM)	17.0 ± 1.4	12.5 ± 1.5†
Daunomycin (100 μM)	27.7 ± 1.2	34.0 ± 1.2
Daunomycin (100 μM) + VP-16 (50 μM)	39.9 ± 2.5	32.2 ± 1.8

* NADPH oxidation and oxygen uptake were carried out as described in Materials and Methods. VP-16 was dissolved in DMSO and added to the incubation mixture (the final concentration of DMSO was 0.1%). The control contained an equivalent amount of DMSO. DMSO, at this concentration, had no effect on NADPH oxidation or oxygen uptake. Values are mean ± S.D. (N = 3 or more).

† For oxygen uptake studies, the concentration of VP-16 was 100 μM.

reductase. In fact, VP-16 stimulated the transfer of electrons. We have also examined the effects of VP-16 on oxygen consumption since VP-16 may inhibit lipid peroxidation by altering reduction of oxygen to reactive species. The data in Table 1 show that VP16 did not alter the basal and daunomycin-promoted oxygen consumption, suggesting that it did not appreciably affect the rate of oxygen metabolism.

The inhibition of the endogenous and daunomycin-induced peroxidation by VP-16 may result from either (a) the decrease or inhibition of daunomycin semiquinone free radical formation or (b) a reaction which scavenges the reactive oxygen-derived toxic species ($\dot{O}_2^-$, $\dot{O}H$) or lipid and lipid peroxy radicals. To evaluate whether VP-16 will affect the formation of daunomycin semiquinone, daunomycin (1 mM) was anaerobically incubated with microsomal proteins in the presence of NADPH. The resultant spectrum of the semiquinone radical had a g-value of 2.0037

and was similar to those previously published [17, 18] during the microsomal incubation with daunomycin. The addition of VP-16 (1 mM) affected neither the rate nor the intensity of the radical formation.

To evaluate whether superoxide anion radical, produced during microsomal-NADPH incubation, may interact directly with VP-16, VP-16 was incubated with xanthine oxidase and xanthine. Xanthine oxidase, widely distributed *in vivo*, produces $\dot{O}_2^-$ in the presence of xanthine [19]. In the incubation mixtures containing VP-16 (1 mM) and xanthine oxidase-xanthine system, no VP-16 radical could be detected, suggesting that VP-16 does not react with $\dot{O}_2^-$ to form a secondary radical. This is consistent with a low rate of reaction observed with O_2^- and other phenolic antioxidants.

VP-16 has been reported to be metabolized by microsomes in the presence of NADPH, and the hydroxyacid derivative, formed from the opening of the lactone ring, has been identified as the major metabolite of VP-16 [20]. Esterases have been implicated in its formation. Furthermore, during microsomal incubation, VP-16 or a metabolite of VP-16 binds irreversibly to the proteins [21]. The identity of the intermediate that binds irreversibly is not known. Since VP-16 contains two methoxyl groups (—OCH_3) at the 3′- and 5′-positions of the phenol ring system (see Fig. 1), O-demethylation catalyzed by cytochrome P-450 is possible. Incubation of VP-16 with microsomes resulted in formation of formaldehyde which was NADPH dependent (Table 2). Furthermore, the formation of formaldehyde was increased in the presence of PB-induced microsomes (Table 2).

Our studies, for the first time, show that VP-16 undergoes O-demethylation during microsomal activation. VP-16, however, is not a good substrate for O-demethylase, as judged by the amount of formaldehyde formed (one-tenth that formed from aminopyrine). Nevertheless, the O-demethylation of VP-16 may be significant and would result in the formation of *o*-dihydroxy (3′,4′-dihydroxyl) derivative, as shown in Ref. 22. The dihydroxy compound could then be enzymatically oxidized to the *o*-quinone. Recently, we have shown that the microsomal metabolism of VP-16 results in the formation of a reactive intermediate that binds irreversibly to proteins and exogeneously added DNA. This binding is NADPH dependent and is increased significantly by PB-induced microsomes [22]. We proposed that this reactive intermediate may be the *o*-quinone metabolite of VP-16 and may thus be important in the cytotoxicity of VP-16.

The present study shows that VP-16 was a powerful inhibitor of microsomal lipid peroxidation. PDP, the parent drug of VP-16, showed only a small inhibitory effect. PDP does not contain a free OH group in the 4′-position of the ring (see Fig. 1), suggesting that 4′-OH of VP-16 may be important in the antioxidant and other biological properties of VP-16. This is consistent with the earlier observations of Loike and Horwitz [4] in that VP-16, but not PDP, was an effective inducer of single- and double-stranded DNA breaks in HeLa cells. The mechanism of the inhibition of the microsomal lipid peroxidation, however, appears to be complex. The daunomycin-promoted lipid peroxidation involves one-electron reduction of the drug by NADPH cytochrome P-450 reductase to its semiquinone free radical [15]. The semiquinone radical is believed to be an obligatory intermediate for the generation of toxic oxygen-derived species for the enhancement of lipid peroxidation [11, 23]. Our studies show that VP-16 did not affect the initial formation of the daunomycin free radical. Furthermore, VP-16 had no effect on the rate of oxygen consumption by daunomycin semiquinone. Our studies also show that VP-16 did not react with or scavenge $\dot{O}_2^-$ generated by xanthine oxidase-xanthine system. These observations, taken together, indicate that the antioxidant effect of VP-16 was not dependent upon formation of daunomycin semiquinone free radical or consequent formation/modifications of $\dot{O}_2^-$ concentrations during microsomal activation of daunomycin. In an experiment designed to investigate the reaction of VP-16 with $\dot{O}H$ generated from H_2O_2 and Fe^{2+} in microsomes, we found that VP-16 did not exhibit any significant inhibition on the formation of DMPO-OH adducts. The lack of inhibition may be due to very low solubility of VP-16 in aqueous medium where the $\dot{O}H$ radicals are generated. However, in lipid medium it can inhibit lipid peroxidation by scavenging $\dot{L}$ and $L\dot{O}O$ in a manner analogous to vitamin E or BHT [24].

The formation of the *o*-dihydroxy- or *o*-quinone of VP-16 may also be implicated in its antioxidation effect. The *o*-dihydroxy derivative may chelate iron or other metal ions that may be required for lipid peroxidation [25]. In this context VP-16 (50 μM) also inhibited the non-enzymic, ascorbate-promoted and enzymatic NADPH-dependent

Table 2. Demethylation of VP-16 and PDP during microsomal incubations in the presence of an NADPH-generating system*

	Rate of demethylation (nmoles HCHO formed 1 hr 1 mg protein)	
	Control	PB-induced
Aminopyrine (2 mM)	577.0 ± 8	1310.7 ± 76.0
p-Nitroanisole (2 mM)	54.2 ± 15	183.4 ± 3.0
VP-16 (2 mM)	42.6 ± 6	65.7 ± 1.0
PDP (2 mM)	18.3 ± 3	29.7 ± 1.0

* Demethylation studies were carried out by incubating the drug with microsomes with or without the NADPH-generating system (NADP 1.0 mM, glucose-6-phosphate and glucose-6-phosphate dehydrogenase 5.0 units/ml) in a total volume of 2 ml at 37°. While aminopyrene and *p*-nitroanisole were incubated for 15 min (which was optimal), VP-16 and PDP were incubated for 30 min. Aminopyrine was dissolved in the buffer. *p*-Nitroanisole was dissolved in ethanol and added to the incubation mixture. PDP and VP-16 were dissolved in polypropylene glycol (mol. wt ~ 200) and added to the incubation mixture. The final concentrations of ethanol and polypropylene glycol were 0.1% and 0.5% respectively. Under these conditions, the vehicles had no effect on the formation of formaldehyde.

microsomal lipid peroxidation (E. G. Mimnaugh, personal communication). Increasing the concentration of VP-16 above 50 μM had no additional inhibitory effect on ascorbate-promoted peroxidation. These preliminary observations suggest that the antioxidant effects of VP-16 may involve either (a) scavenging of lipid or lipid peroxy radicals, and/or (b) chelation of metal ions by the drug or by a metabolite formed by microsomal oxidation. Currently, we are attempting to synthesize the *o*-dihydroxy derivative and evaluate its biological properties.

In summary, incubation of VP-16 with mouse liver microsomes in the presence of NADPH resulted in O-demethylation which was enhanced by phenobarbital-induced microsomes. This O-demethylation may be significant since it would result in the formation of the *o*-dihydroxy derivative of VP-16 which may then be subsequently oxidized to the respective quinone. Such a quinone derivative of VP-16 could bind to critical cellular macromolecules causing cytotoxicity. VP-16 was also a potent dose-dependent inhibitor of both the basal and daunomycin-promoted microsomal lipid peroxidation. By contrast, podophyllotoxin, the parent compound, had very little effect on lipid peroxidation, suggesting that the 4′-OH group of VP-16 was necessary for this activity.

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Hydrogen bond structure in the glucocorticoid agonist–receptor complex

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The interaction between various steroids and the glucocorticoid receptor is largely determined by van der Waals interactions [1]. In addition, 11β hydroxysteroids form a hydrogen bond with the receptor as we have shown recently [2]. This hydrogen bond appears to have considerable functional significance: its formation may render a conformation of the complex preferable, starting from which it may easily undergo activation. This is probably why most of the optimal glucocorticoids have an 11β-hydroxyl group [3].

The oxygen atom of that hydroxyl may in principle, donate as well as accept, a hydrogen atom when participating in a hydrogen bond. To find out how the hydrogen bond is directed we used in this study two steroids that have 11β-chloro- rather than 11β-hydroxy-substituents and can thus be acceptors, but not donors, in hydrogen bonds.

Materials and methods

Chemicals, media and buffers. [1,2^{3}H]Triamcinolone acetonide (26 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, U.K. Dichlorisone and 16α-methyl dichlorisone were donated by the Steroid Reference Collection, Hampstead, London (Curator: Prof. D. N.