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IN VITRO METABOLISM OF ETOPOSIDE (VP-16-213) BY LIVER MICROSOMES AND IRREVERSIBLE BINDING OF REACTIVE INTERMEDIATES TO MICROSOMAL PROTEINS*

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Abstract—We have studied the metabolism of VP-16-213 (etoposide, VP-16), an antitumor agent, by mouse liver microsomes to reactive intermediates and the subsequent covalent binding to microsomal proteins. This metabolism was shown to involve the O-demethylation of VP-16 and resulted in the formation of a 3′,4′-dihydroxy derivative (DHVP-16) which was identified by both HPLC and mass spectrometry. The formation of DHVP-16 was cytochrome P-450-mediated as indicated by its dependence on NADPH, its increased production following treatment of mice with phenobarbital, and its marked inhibition by SKF-525A and piperonyl butoxide. Furthermore, DHVP-16 formation required oxygen. Microsomal incubation of VP-16 resulted in an irreversible binding of the drug to the proteins, which was also shown to be cytochrome P-450 dependent. The covalent binding of the VP-16 metabolite(s) was inhibited by DHVP-16 in a dose-dependent fashion, suggesting that the reactive intermediates that bound to proteins were derived from DHVP-16. Electron spin resonance studies indicated that the same semiquinone radical was formed during enzymatic (oxidation or reduction) metabolism of DHVP-16 and the *o*-quinone derivative of VP-16 (VP-16-Q). VP-16-Q and its semiquinone radical are suggested to be the bioalkylating species.

The semisynthetic podophyllotoxin derivative VP-16-213¶ (VP-16, etoposide, Fig. 1) has been demonstrated to be active in the treatment of several neoplastic disorders such as small cell lung cancer, testicular tumors, and malignant lymphomas [1, 2]. VP-16 has been shown to induce both single- and double-strand DNA breaks in several tumor cell lines *in vitro* [3–8] and also in isolated tumor cell nuclei [5]. Since VP-16 does not induce DNA when incubated with purified DNA *in vitro* [3], it is likely that some other cellular components are required for its pharmacological effects. We have reported recently that the incubation of VP-16 with liver microsomes results in NADPH-dependent O-demethylation of the drug, as determined by formaldehyde formation [9]. The metabolism of VP-16 has also been shown to result in an irreversible binding of the drug to microsomal proteins [10, 11] and to exogenously added calf thymus DNA [10]. However, the nature of the reactive metabolite formed during microsomal metabolism which bioalkylates cellular macromolecules is unknown at this time. We have, therefore, further characterized the microsomal metabolism of VP-16, and we report here on the formation of a novel metabolite, DHVP-16, which has been confirmed by HPLC and mass spectrometry. We also present evidence that the covalent binding of VP-16 to microsomal proteins is dependent upon DHVP-16 formation. The present study shows that both the formation and the covalent binding of these reactive intermediates to proteins were cytochrome P-450 dependent. These events may be important in the cytotoxicity of the drug.

MATERIALS AND METHODS

VP-16 was a gift from Bristol-Myers Pharmaceuticals, Syracuse, NY. [^{3}H]VP-16 (200 mCi/mmole) was obtained from Moravek Biochemicals Inc., Brea, CA, and its radiochemical purity was 98% as determined by reverse phase HPLC analysis. NADPH was obtained from the United States Biochemical Corp., Cleveland, OH. Reduced glutathione, phenobarbital sodium salt, horseradish peroxidase (RZ = 3.0), glucose-6-phosphate, glucose-6-phosphate dehydrogenase and polyethylene glycol ($M_r \sim 200$) were from the Sigma Chemical Co., St. Louis, MO. SKF-525A was a gift from Smith Kline & French Inc., Philadelphia, PA. Piperonyl butoxide was obtained from Fluka Chemicals. DHVP-16 and VP-16-Q derivatives were synthesized (Nemec *et al.*, to be published). The standards were dissolved in methanol and stored at −70°.

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¶ Abbreviations: VP-16-213 (VP-16), 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside); DHVP-16, 3′,4′-dihydroxy-VP-16; VP-16-Q, *o*-quinone derivative of VP-16; HPLC, high performance liquid chromatography; SKF-525A, β-diethylaminoethyl-diphenylpropyl acetate·HCl; ESR, electron spin resonance; HRP, horseradish peroxidase; and DETAPAC, diethylenetriaminepentaacetic acid.

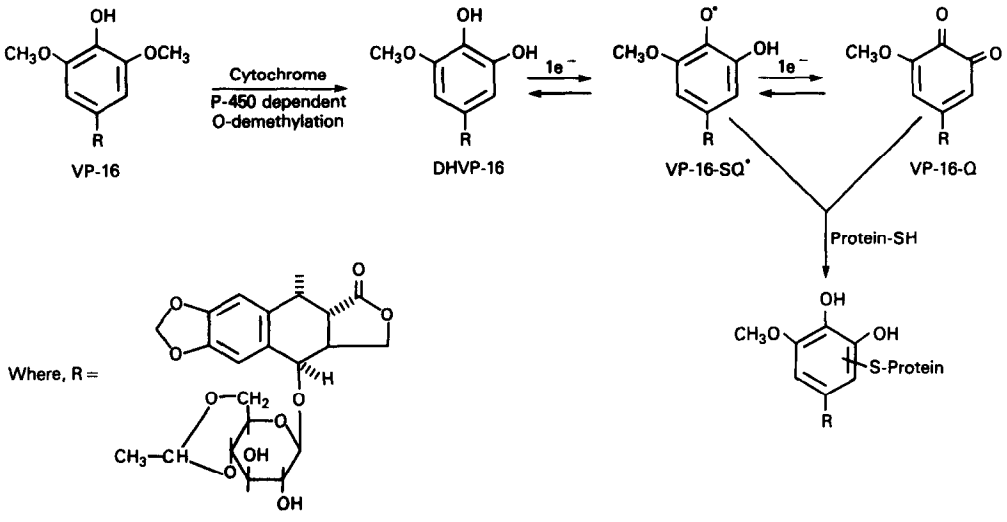

Fig. 1. Structures of VP-16, DHVP-16 and VP-16-Q and proposed cytochrome P-450-dependent metabolism of VP-16 resulting in the formation of VP-16-Q and VP-16 semiquinone free radical (VP-16-SQ·) which can bind to proteins and DNA.

Purified NADPH–cytochrome P-450 reductase was prepared according to the method of Shepard *et al.* [12] and was a gift of Dr. G. Krishna. Hepatic microsomes were prepared from adult CD_2F_1 male mice according to a previously published method [13]. Pooled liver microsomal proteins were stored at −70°. Unless otherwise indicated, mice were treated with phenobarbital (80 mg/kg, i.p., ×3 daily). Food and water were provided *ad lib.*, and mice were killed by cervical dislocation. Protein was determined according to the method of Lowry *et al.* [14] with bovine serum albumin as a standard.

Incubation conditions. All microsomal incubations were carried out in 150 mM KCl–50 mM Tris (pH 7.4) at 37°. The buffer was bubbled with oxygen (10–15 min). VP-16, dissolved in polyethylene glycol (final volume 0.5%), was added to microsomes and mixed on ice for 5–10 min. The mixture was warmed to 37° for 2 min, and the reaction was initiated by adding NADPH (2 mM). The mixture was continuously shaken and bubbled with oxygen every 20 min. For the anaerobic incubations, nitrogen was bubbled for 10 min, and the samples were sealed under nitrogen. All reactions were terminated by adding 5 ml of chloroform on ice followed by immediate shaking of the mixtures.

HPLC analysis. For HPLC analysis for the detection of metabolites, aliquots (1 ml) of microsomal incubations were shaken for 15 min with 5 ml chloroform. Following centrifugation, the organic layer was separated and the aqueous layer was reextracted with chloroform (3 ml). The combined organic layers (8 ml) were collected and evaporated under a gentle stream of nitrogen at room temperature, and the residues were dissolved in 200 μl of ice-cold methanol, filtered with a 0.2 μm filter, and 20 μl was injected into the HPLC column. To determine the extraction recovery, known quantities of authentic DHVP-16 were added to microsomes, immediately extracted, and analyzed by HPLC.

The mobile phase for the HPLC analysis consisted of 60% methanol and 40% water (v/v, "solvent A"). In some experiments acetonitrile (30%) and 0.01 M sodium acetate (70%, v/v, pH adjusted to 3.8 with glacial acid, "solvent B") were also used. The solvent was pumped via a Rainin Rabbit-HP delivery system isocratically at 1 ml/min, through a Bondapak Phenyl column. An LKB detector was used to monitor the absorbance at 277 nm.

Mass spectrometer analysis of VP-16 metabolite. A VG Micromass ZAB-2F mass spectrometer (VG Instrument, Manchester, England) operating in electron impact (EI) ionization and double-focusing mode was used for mass spectrometric analysis. The following spectrometer settings were used: electron energy = 70 eV; trap current = 100 μA; emission current = 300 μA; source temperature = 180° with an exponential down scan from mass 700 to 50 at a 2 sec/decade rate and reset time of 1 sec. The samples, dissolved in methanol–water, were introduced via a standard VG Instrument thermal desorption probe and were allowed to evaporate on the probe wire before introduction into the source. The current to desorb the sample was applied manually to obtain a steady ion current of the peak interest. The spectra presented here have been background subtracted via a nominal mass background subtraction routine with the mass spectrometer data system. The five most intense molecular ion spectra were then averaged together to give the resulting spectra.

The ESR study for the detection of semiquinone radicals from either DHVP-16 or VP-16-Q was carried out on a Varian E-104 spectrometer in 10 mM phosphate-buffered saline containing 0.1 mM DETAPAC (pH 7.4) at room temperature.

Covalent binding of VP-16. The irreversible binding of VP-16 and/or its metabolites to the microsomal proteins was determined in an incubation mixture containing [^{3}H]VP-16 (0.5 μCi/ml) in methanol

(final volume 0.1%) diluted with non-radioactive drug. The removal of the unbound drug from the proteins was achieved as follows: 5 ml of chloroform were added to 5-ml aliquots of the microsomal incubation mixtures, followed by extraction by shaking for 15 min, and centrifugation, and the organic layer was discarded. The above procedure was repeated with an additional 5 ml of chloroform, and the proteins were precipitated with 2 ml of 50% trichloroacetic acid. The precipitated protein was collected by centrifugation, washed repeatedly with the KCl–Tris buffer (10 ml × 2) and methanol (10 ml × 3). The protein pellet was dried with a stream of nitrogen and dissolved in 5 ml of 1% sodium dodecyl sulfate by sonication for 45 min. The protein was reprecipitated with 50% trichloroacetic acid (2 ml) and collected by centrifugation; the pellet was washed repeatedly with the KCl–Tris buffer (5 ml × 2) and methanol (5 ml × 3). Under these conditions of isolation, no further radioactivity could be removed from the proteins. The samples were dissolved in 2 ml of 0.5 N sodium hydroxide by heating to 60° in a water bath for 20 min, aliquots (70 μl) were removed for protein assay, and 1 ml was transferred to a scintillation vial containing 0.5 ml of 1 N HCl, to which 10 ml of scintillation fluid (Hydrofluor) was added. The radioactivity bound to proteins was determined using a Searle 6880 liquid scintillation counter (Mark III), and appropriate quenching corrections were made. The data are expressed as nmoles of VP-16 and/or metabolite bound per mg protein.

Effects of inhibitors. The effects of inhibitors on the microsomal metabolism were studied under similar conditions except that the microsomes were preincubated with the inhibitors for 10 min on ice. SK-525A and glutathione were dissolved in the KCl–Tris buffer. Piperonyl butoxide was dissolved in acetone, and DHVP-16 in polyethylene glycol (final volumes of the above vehicles were 0.5%, v/v). Under these conditions, the vehicles had no effect on either VP-16 metabolism or drug–protein binding.

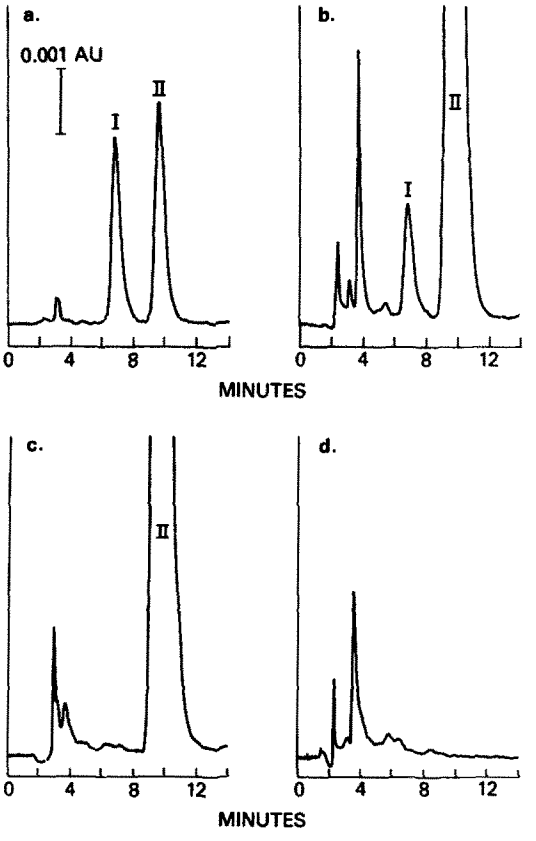

Fig. 2. HPLC chromatograms with "solvent A" obtained from (a) authentic DHVP-16 (I), 1.5 μg, and VP-16 (II), 2.1 μg, with retention times of 6.8 and 9.6 min, respectively, (b) the chloroform extract of the microsomal incubation (1 ml) containing 1 mg/ml protein, VP-16 (500 μM) and 2 mM NADPH, (c) same as (b) except it contained no NADPH, and (d) same as (b) except it contained no VP-16. The absorbance was measured in an attenuation of 0.01 absorbance units (AU), full scale.

RESULTS

HPLC analysis of the chloroform extracts of microsomal incubations of VP-16 in the presence of NADPH, revealed a peak with a retention time identical to that of DHVP-16 in either "solvent A" (Fig. 2, a and b) or "solvent B" (data not shown). When the incubations were carried out in the absence of either NADPH or VP-16 (Fig. 2, c and d respectively) or with heat-inactivated microsomes (20 min at 80°), no peak corresponding to this metabolite was present. No other metabolites were observed by HPLC. However, when the chloroform extract of the microsomal incubations containing VP-16 and NADPH was exposed to air at room temperature or when the aqueous solution of DHVP-16 was treated with a catalytic amount of Fe^{3+}, a peak corresponding to VP-16-Q (retention time of 7.1 min, eluted with the "solvent A" only) was detected, suggesting that DHVP-16 was autooxidized to the quinone derivative. Furthermore, the quinone was rapidly reduced to DHVP-16 when treated with NADPH.

The identity of the metabolite with the same retention time as that of DHVP-16 was further confirmed by mass spectrometry (Fig. 3). Figure 3A shows the mass spectrum of the authentic DHVP-16 and Fig. 3B compares the mass spectrum of the metabolite formed by incubating VP-16 with the complete microsomal system. Both spectra show molecular ions at m/z 574 and a base peak at m/z 368 which results from the loss of the sugar moiety. The peak at m/z 366 present in the mass spectra represents the quinone form of VP-16, VP-16-Q, which may form from the partial autooxidation of DHVP-16.

Using HPLC conditions as described in Fig. 2, standard curves were determined for DHVP-16 by adding known amounts of the compound to microsomes and extracting with chloroform as described above. The extraction recovery for DHVP-16 in the concentration range of 1 to 33 μg/ml was 97 ± 2%.

Taking into account the assay sensitivity, the following experimental conditions were chosen: VP-16, 500 μM; microsomal protein, 1 mg/ml; and incubation period, 1 hr. The effect of varying the protein concentration on DHVP-16 formation was linear up

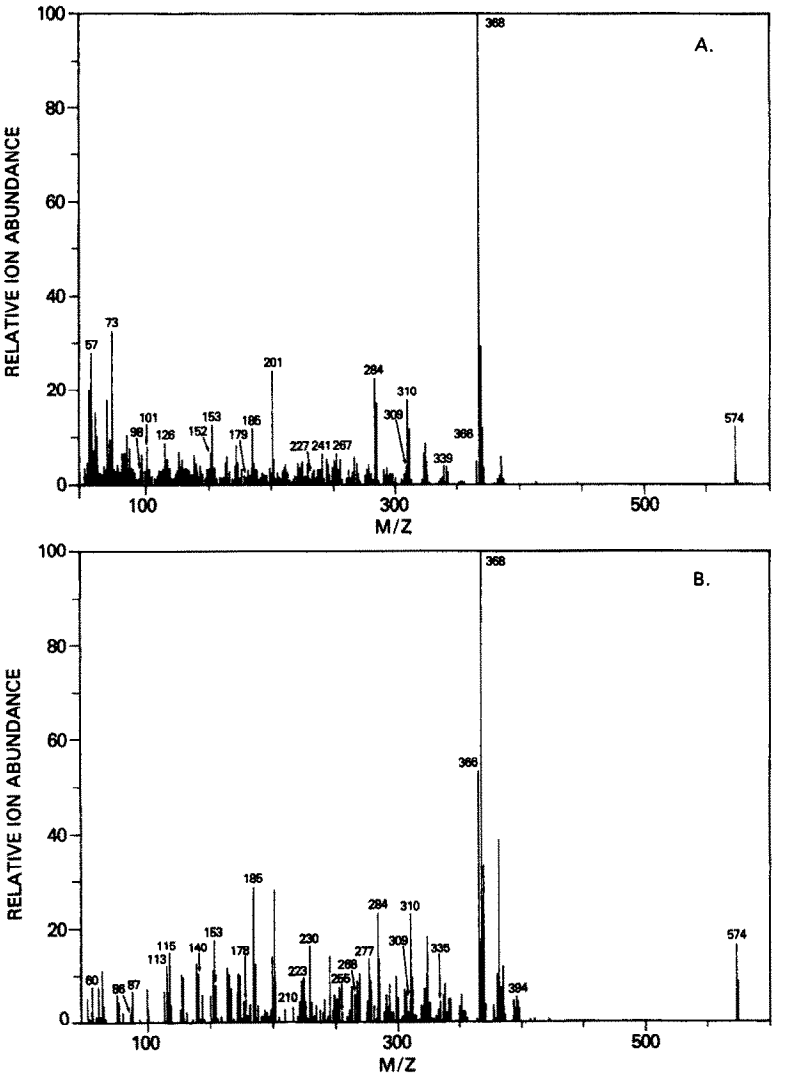

Fig. 3. Electron impact mass spectra obtained from: (A) the authentic DHVP-16 and (B) the metabolite with the retention time identical to that of DHVP-16. The microsomal incubation containing VP-16 and NADPH was extracted with chloroform, and the relevant peak was collected by HPLC with "solvent A" and further extracted with chloroform. The residue was dissolved in methanol and analyzed.

to 1 mg/ml microsomal protein, and was saturated at about 2 mg/ml (Fig. 4). The formation of DHVP-16 was time dependent, as shown in Fig. 5. It was linear up to about 15 min and began to level off after 1 hr of incubation. A similar time course was also obtained when an NADPH-generating system (NADPH, 1 mg/ml; glucose-6-phosphate, 2 mg/ml; and glucose-6-phosphate dehydrogenase, 5 units/ml) was used. The formation of DHVP-16 was also dependent on the VP-16 concentration (Fig. 6). Under these experimental conditions, the apparent K_m for the reaction was found to be 378 μM. Within the linear part of the concentration-dependent curve (i.e. up to 100 μM VP-16), 4.6% of the parent drug was converted to DHVP-16. Further characterization of VP-16 metabolism to DHVP-16 under different experimental conditions is presented in Table 1. The reaction was oxygen dependent and was enhanced by phenobarbital induction. The known inhibitors of cytochrome P-450, SKF-525A and piperonyl butoxide [15, 16], markedly inhibited DHVP-16 production (Table 1). In addition, the formation of DHVP-16 was inhibited by reduced glutathione.

The incubation of microsomal proteins with [^{3}H]VP-16 resulted in irreversible binding of the radiolabel to proteins, and this binding was markedly greater in the presence of NADPH. The time course of the binding is shown in Fig. 7. Without NADPH, there was only a slight increase in the covalent binding of the drug to the proteins up to 2 hr of incubation. In the presence of NADPH or the NADPH-generating system, the binding increased with time such that after 1 hr of incubation about 2% of the total radioactivity became irreversibly bound representing 2 nmoles of VP-16 and/or its metabolite

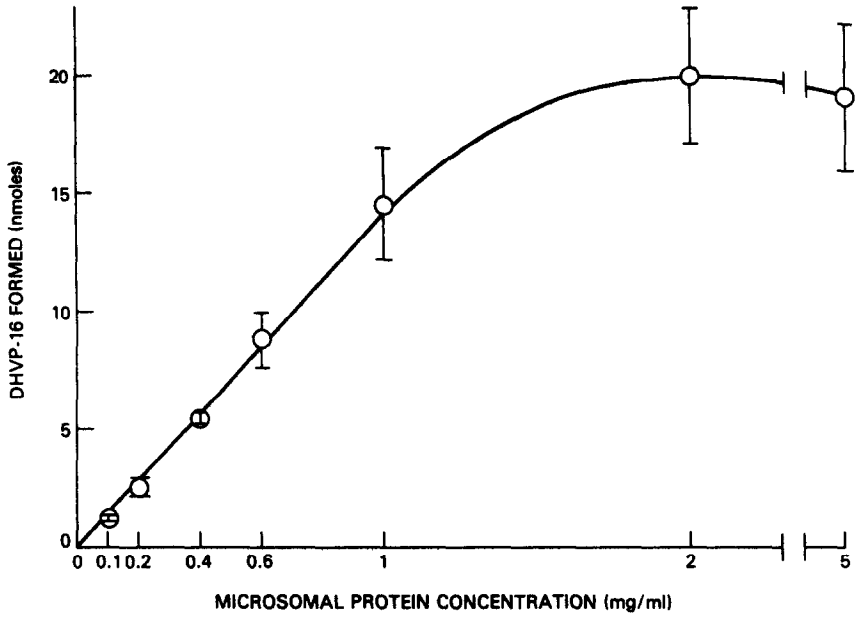

Fig. 4. Dependence of DHVP-16 formation from VP-16 on microsomal protein concentration. The incubations contained 500 μM VP-16, 2 mM NADPH and protein. The chloroform extracts were analyzed for DHVP-16 by HPLC after 60 min of incubation at 37°. The total amount of metabolite formed in a 1-ml incubation is shown. These points represent mean ± SD (bars) of three separate experiments done in duplicate.

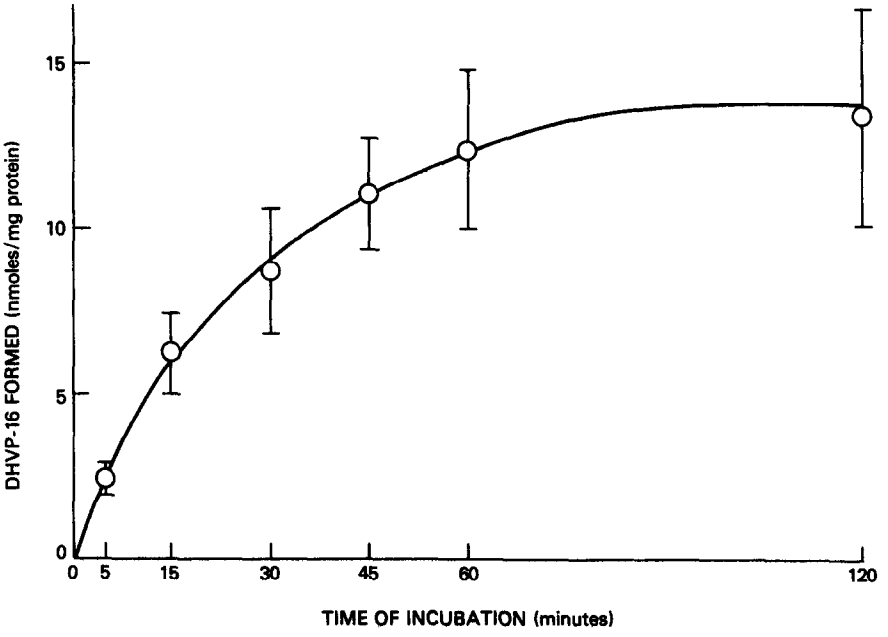

Fig. 5. Time dependence of DHVP-16 formation from VP-16 (500 μM) during incubation with microsomes (1 mg/ml) in the presence of 2 mM NADPH. These points are mean ± SD of three separate experiments done in duplicate.

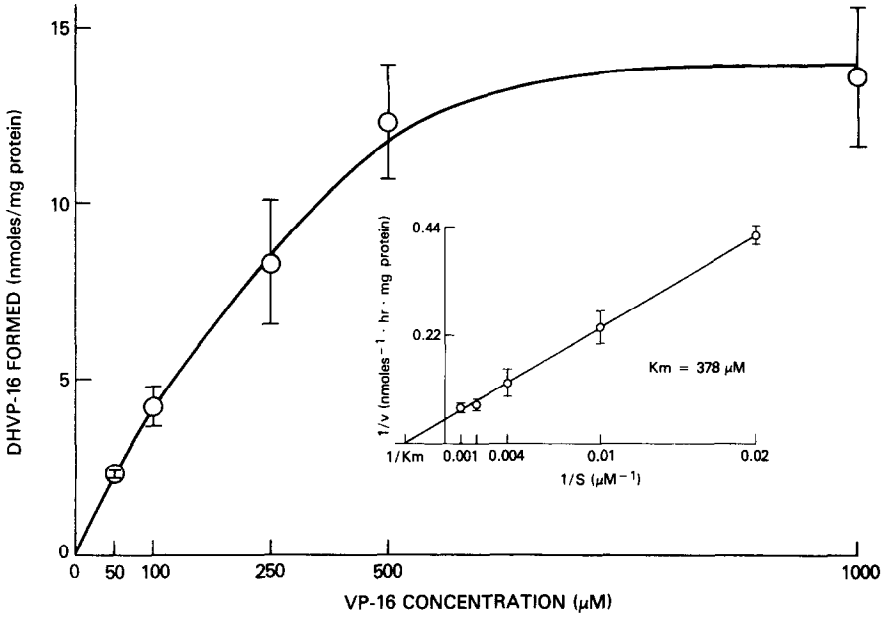

Fig. 6. Dependence of DHVP-16 formation on VP-16 concentration during incubation with microsomes (1 mg/ml) in the presence of 2 mM NADPH at 37° for 60 min. These points are mean ± SD of three separate experiments done in duplicate. Insert: double-reciprocal plot: K_m = 378 μM.

per mg of protein. The binding in the presence of NADPH was higher than in the absence of NADPH with different concentrations of VP-16 as shown in Fig. 8. The characteristics of this binding are presented in Table 2, and they are similar to those for DHVP-16 formation. The binding of VP-16 was oxygen dependent, enhanced by phenobarbital induction, and was inhibited markedly by cytochrome P-450 inhibitors and by reduced glutathione. The irreversible binding of VP-16 was also decreased in the presence of DHVP-16 in a concentration-dependent fashion (Table 2).

To demonstrate that DHVP-16 and VP-16-Q were converted to semiquinone radicals which could bind to proteins during the metabolism of VP-16, ESR studies were initiated. The incubation of DHVP-16 with HRP (1 mg/ml) and H_2O_2 (400 M) was observed to rapidly form the semiquinone radical (data not shown). In the presence of microsomes and NADPH, however, only a trace of the radical was detected (data not shown). Since cytochrome P-450 has been shown to act as peroxidase, and can catalyze the one-electron oxidation of xenobiotics [17, 18], H_2O_2 was substituted for NADPH in the incubation mixtures containing microsomes and DHVP-16. Figure 9A shows the resulting ESR spectrum of the semiquinone radical formed in the presence of H_2O_2. The addition of reduced glutathione to the incubation mixtures inhibited the formation of the radical intermediate, suggesting that glutathione reduced this radical and/or bound to it (Fig. 9B). VP-16-Q also formed the same semiquinone radical

Table 1. Characteristics of DHVP-16 formation from VP-16 under various conditions

Condition	(nmoles DHVP-16 formed*/mg protein)	% of control
Control†	12.87 ± 2.35	
No NADPH	ND‡	
Non-induced microsomes	5.82 ± 1.80§	45
Nitrogen	1.80 ± 0.48§	14
SKF-525A (0.5 mM)	2.65 ± 0.74§	21
Piperonyl butoxide (1 mM)	3.68 ± 0.87§	29
Reduced glutathione (1 mM)	3.98 ± 1.21§	31

* Mean ± SD of three to nineteen separate experiments done in duplicate and analyzed by HPLC.

† VP-16 (500 μM) was incubated with phenobarbital-induced microsomes (1 mg/ml) in the presence of NADPH (2 mM) at 37° for 1 hr.

‡ Not detected.

§ Significantly different from control ($P < 0.001$).

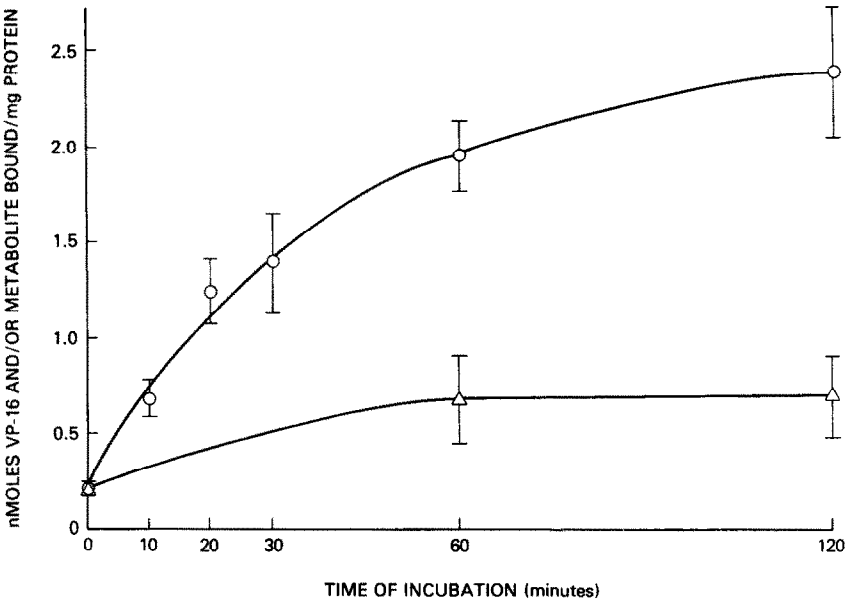

Fig. 7. Time course of VP-16 and/or metabolite binding to microsomal proteins. The protein (1 mg/ml) was incubated with VP-16 (100 μM) containing [^{3}H]VP-16 (0.5 μCi/ml) in the presence (○—○) and absence (△—△) of 2 mM NADPH at 37° for 60 min. The results are expressed as total drug or metabolite bound/mg protein and are mean ± SD of three separate experiments done in duplicate.

when incubated with NADPH and either purified NADPH-cytochrome p-450 reductase or liver microsomes (Fig. 9, C and D, respectively). Under identical conditions, however, a 30–40% decrease in the amount of the radical was detected in the presence of microsomes, indicating that either the quinone or the semiquinone radical bound to the microsomal proteins (compare Fig. 9, C and D). In addition, the radical intermediate was also formed (non-enzymatically) from VP-16-Q at pH 8 (data not shown), a reaction common to quinones including adriamycin [19]. The addition of microsomes to this preformed radical decreased its signal intensity, suggesting the binding of the radical intermediate to the microsomal proteins.

DISCUSSION

While VP-16 is metabolized *in vivo* [20–23], its metabolism remains poorly defined. The major metabolite of VP-16, identified in humans, is the hydroxy acid [21, 22], formed as a result of hydrolysis

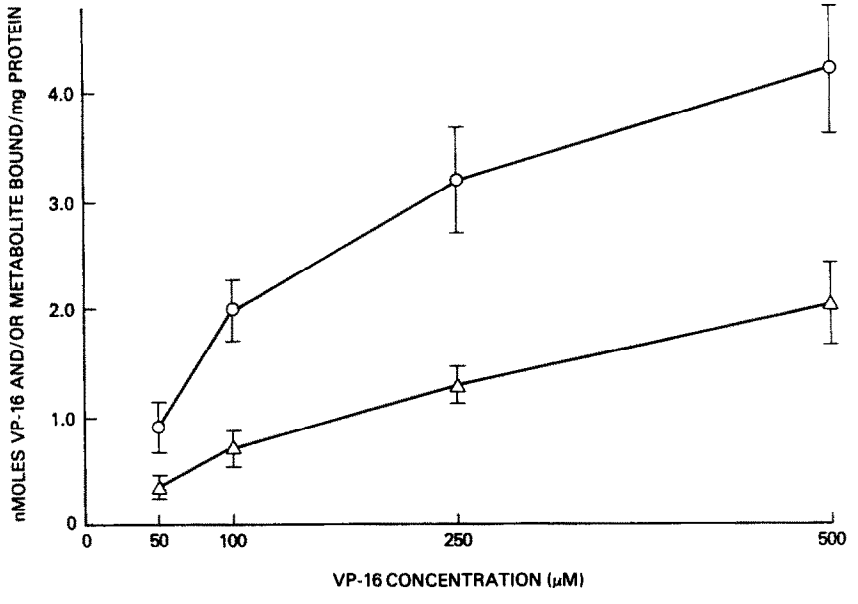

Fig. 8. Dependence of protein binding on VP-16 concentration. The protein (1 mg/ml) was incubated in the presence (○—○) or absence (△—△) of 2 mM NADPH at 37° for 60 min.

Table 2. Characteristics of irreversible binding of VP-16 and/or metabolite to microsomal proteins under various conditions

Condition	(nmoles Binding/mg protein)*	% of Control
Control†	1.97 ± 0.23	
No NADPH	0.67 ± 0.20‡	34
Non-induced microsomes	0.61 ± 0.24‡	31
Nitrogen	0.50 ± 0.19‡	25
SKF-525A (0.5 mM)	0.78 ± 0.03‡	40
Piperonyl butoxide (1 mM)	0.74 ± 0.13‡	38
Reduced glutathione (1 mM)	0.43 ± 0.07‡	22
DHVP-16		
10 μM	1.65 ± 0.21	84
50 μM	1.03 ± 0.09‡	52
100 μM	0.82 ± 0.11‡	42

* Mean ± SD of three to six separate experiments done in duplicate.
† VP-16 (100 μM) was incubated with phenobarbital-induced microsomes (1 mg/ml) in the presence of NADPH (2 mM) at 37° for 1 hr.
‡ Significantly different from control ($P < 0.001$).

of the trans-γ-lactone ring and has been shown to be biologically inactive *in vitro* [24]. This product was also formed *in vitro* by rat liver microsomes [25]. The hydroxy acid derivative is chloroform insoluble [21] and, thus, was not detected in our HPLC analysis. A number of other metabolites of VP-16 have also been detected in humans and include the *cis*-picro-lactone isomer [22] and the glucuronic acid conjugate [26]. Recently, we have also shown that VP-16 undergoes O-demethylation during *in vitro* peroxidase metabolism to VP-16-Q [27].

The data presented in this manuscript show that the microsomal metabolism of VP-16 also formed a DHVP-16 derivative as a result of O-demethylation. The formation of this metabolite was confirmed by comparison with an authentic sample using both HPLC and mass spectrometric analysis. It is interesting to note that DHVP-16 was readily oxidized (enzymatically and chemically) to VP-16-Q which, in turn, was rapidly reduced to DHVP-16 by NADPH. Moreover, VP-16-Q could not be recovered quantitatively when added to heat-inactivated microsomes, indicating the binding of the quinone to the proteins. Thus, the failure to detect the quinone derivative in microsomal incubations containing NADPH may be due to this rapid reduction and binding. The metabolism of VP-16 to DHVP-16 was oxygen- and NADPH-dependent, and

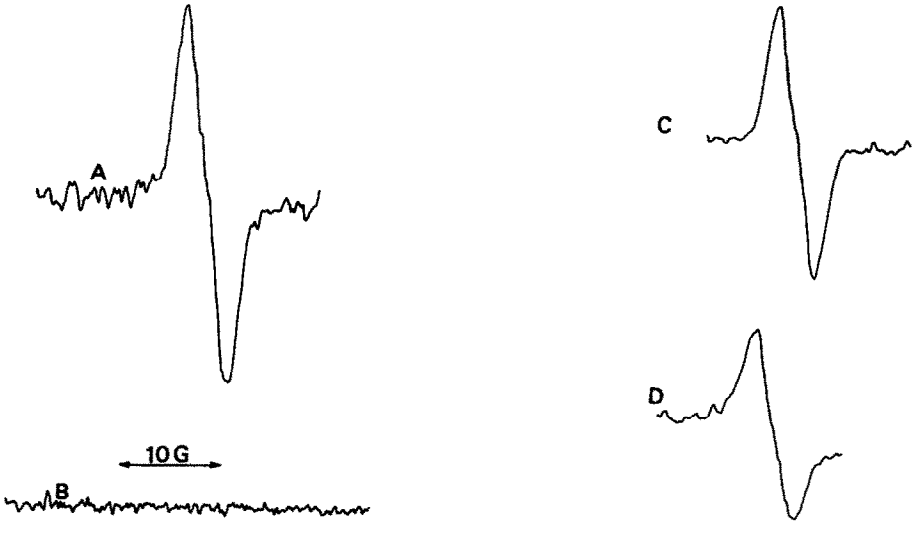

Fig. 9. ESR spectra obtained from (A) DHVP-16 (1 mM) in the presence of liver microsomes (1 mg/ml) and H_2O_2 (500 μM) in 10 mM phosphate-buffered saline containing 0.1 mM DETAPAC; (B) same as (A) except that reduced glutathione (5 mM) was also present; (C) VP-16-Q (1 mM) and 0.2 units/ml purified NADPH–cytochrome P-450 reductase and NADPH (0.1 mM) and (D) VP-16-Q (1 mM) in the presence of liver microsomes (2 mg/ml) and NADPH (0.1 mM). The ESR settings were: field = 3390 G; microwave power = 20 G; and modulation amplitude = 2.0 G; receiver gain was 1×10^5 for (A) and (B) and 2.5×10^4 for (C) and (D).

it was enhanced by phenobarbital induction and markedly inhibited by SKF-525A and piperonyl butoxide. These results indicate that this metabolic pathway was cytochrome P-450 mediated. DHVP-16 formation was also decreased by the presence of reduced glutathione. This would suggest that glutathione reacted with an intermediate during DHVP-16 formation. VP-16 forms a phenoxy radical during peroxidase activation [28], and if this intermediate is a prerequisite for DHVP-16 formation, glutathione could reduce the phenoxy radical back to the parent drug, thus effectively reducing the accumulation of DHVP-16.

The microsomal incubation of [^{3}H]VP-16 resulted in an irreversible binding of the drug to microsomal proteins. The binding was enzymatic as suggested by the significant increase in the presence of NADPH. Other features (i.e. oxygen dependence, increased binding following phenobarbital induction, inhibition by cytochrome P-450 inhibitors and by glutathione) were similar to those described for DHVP-16 formation. Therefore, it is likely that the irreversible binding of a VP-16 intermediate to microsomal proteins was also cytochrome P-450 mediated. Inhibition of the irreversible binding by glutathione and the effect of glutathione on DHVP-16 formation could result from similar mechanisms. In addition, glutathione may bind to reactive intermediates causing decreased protein binding of the drug. DHVP-16 inhibited the binding of VP-16 metabolites to proteins in a concentration-dependent fashion. Because of the above observation and because of the similarities between the two processes, it is possible that the reactive intermediates, which covalently bind to proteins as the result of the microsomal metabolism of VP-16, are derived from DHVP-16. We have shown previously that VP-16 also binds covalently to DNA following microsomal metabolism [10], and it is possible that the alkylating intermediates are derived from the same metabolic pathway.

Based on the present data, we propose that VP-16 is metabolized by cytochrome P-450 to a reactive intermediate(s) which binds covalently to proteins and DNA, and which may subsequently lead to cellular injury and death (Fig. 1). Since *o*-quinones have been shown to be extremely reactive towards proteins and DNA [29], it is possible that VP-16-Q may be one of the alkylating species. In addition, the semiquinone free radical intermediate generated from either DHVP-16 or VP-16-Q by one-electron oxidation/reduction may also bind to critical targets (Fig. 1). This is supported by our ESR studies which show that both DHVP-16 and VP-16-Q generated the same semiquinone radical, and that microsomal proteins decreased the radical production presumably as a result of binding of the radical to the proteins. The proposed formation of reactive intermediates and their subsequent binding are also consistent with the fact that the recovery of VP-16 given to humans is incomplete, and this is thought to be due to sequestration of metabolites [20].

The liver microsomal system used in our study is a useful model to study the cytochrome P-450-dependent metabolism of VP-16. The metabolism of VP-16 to reactive intermediates could be accomplished by endoplasmic reticulum or nuclear membrane cytochrome P-450 activity in target cells. In this context, the finding of irreversible binding of [^{3}H]VP-16 to HeLa cells microsomal fractions [11] should be mentioned. Alternatively, the O-demethylation of VP-16 could be catalyzed by peroxidases such as prostaglandin synthetase, as we recently reported [27], although the relative contribution to the metabolism of VP-16 by these two pathways is not known.

Metabolic activation of antitumor drugs to reactive species by cytochrome P-450 mixed-function oxidase activity is well documented for a number of antitumor agents, i.e. cyclophosphamide [30] and hexamethylmelamine [31]. The microsomal metabolism of VP-16 to intermediates which bind covalently to proteins and DNA indicates that these metabolites are reactive, potentially damaging, and may be capable of causing cellular injury. It is possible that the hepatotoxicity of VP-16 given at high doses to humans [32] may be related to its metabolism to reactive intermediates. It is also noteworthy that VP-16-Q was found to be biologically active, and preliminary results indicate that VP-16-Q and the related *o*-quinone of VM-26, another clinically active podophyllotoxin, are highly active *in vivo* against murine leukemia L1210 cells [33]. Van Maanen *et al.* [34] have shown recently that biologically active ϕX174 DNA is inactivated by VP-16-Q but not by VP-16 itself. These observations strongly suggest that a metabolically activated form of VP-16 may participate in its antineoplastic activity.

Several investigators have proposed that type II topoisomerase is a likely intracellular target for the DNA strand-breaking effects of VP-16 and that the induction of DNA damage is related to the cytotoxicity of the drug [5–8]. It is not clear at this time how the metabolism of VP-16 and subsequent binding of reactive species to proteins and DNA are related to the cytotoxicity of VP-16.

In conclusion, we have shown that VP-16 undergoes O-demethylation, catalyzed by a microsomal cytochrome P-450, to form reactive intermediates which bind to cellular constituents. The exact role of the cytochrome P-450-mediated metabolism of VP-16 in its antitumor properties remains to be elucidated, especially in the *in vivo* situation. Assessment of the potential antitumor activity of the newly discovered metabolites (DHVP-16 and VP-16-Q) also deserves special attention.

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