Eur J Cancer Clin Oncol, Vol. 21, No. 9, pp. 1099–1106, 1985.
Printed in Great Britain.

0277-5379/85$3.00+0.00

The Role of Metabolic Activation by Cytochrome P-450 in Covalent Binding of VP 16-213 to Rat Liver and HeLa Cell Microsomal Proteins*

J. M. S. VAN MAANEN,†‡ C. DE RUITER,§ J. DE VRIES,§ P. R. KOOTSTRA,§ F. GOBAS§ and H. M. PINEDO†

†*Department of Oncology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands and* §*Section on Molecular Toxicology, Department of Pharmacochemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*

Abstract—*Covalent binding of* ^{3}H-*labeled VP* 16-213 *to rat liver and HeLa cell microsomal proteins was studied* in vitro. *Metabolic activation by cytochrome P-450 was found to play a role in the covalent binding of VP* 16-213 *to rat liver microsomal proteins, as shown by the need of NADPH cofactor, the increased binding after phenobarbital pretreatment and the inhibition by SFK-525A. Addition of ascorbic acid or α-phenyl-N-tert. butylnitrone to the incubation mixture depressed covalent binding by about* 85%, *suggesting that formation of a reactive metabolite from the phenolic structure may be involved in the binding process. VP* 16-213 *did not inhibit aminopyrine N-demethylase at the concentration used in the binding experiments* (17 μM), *indicating that metabolism of its methylenedioxy group does not play a role in binding to microsomal proteins. HeLa cell microsomes were found to possess aminopyrine N-demethylase activity. Covalent binding of radiolabeled VP* 16-213 *to HeLa cell microsomes decreased by about* 64% *if NADPH was omitted.*

INTRODUCTION

VP 16-213 [4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D- glucopyranoside), NSC 141540] (Fig. 1) is an important anti-neoplastic agent used against a variety of tumors [1]. Few studies have investigated its mechanism of action. Loike and Horwitz [2] studied its effect on HeLa cell DNA and concluded that VP 16-213 caused DNA single-strand breaks. Exposure of isolated purified DNA to VP 16-213 did not lead to breaks, which suggests that activation of one or several endonucleases or transformation into a reactive metabolite is responsible for the effect on HeLa cell DNA, rather than direct chemical cleavage. More recently, Wozniak and Ross [3] have suggested that activation of the drug is required for its effect on DNA.

The approach required to determine whether a given reaction is mediated by a chemically reactive metabolite includes the covalent binding of radioactive metabolites to proteins and other tissue components after the addition of radiolabeled drugs [4, 5]. We applied this principle to study the role of oxidative metabolic activation in covalent binding of VP 16-213 to rat liver and HeLa cell microsomal proteins.

Accepted 22 March 1985.

*This study was supported by the Netherlands Cancer Foundation (grant No. I.K.A. 81-15).

‡To whom requests for reprints should be addressed at: Department of Oncology, Room V-1066A, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.

MATERIALS AND METHODS

Covalent binding to rat liver microsomal proteins

Chemicals. VP 16-213 labeled with tritium at position C-1 (sp. act. 58.92 mCi/mmol) and unlabeled VP 16-213 were gifts from the Bristol Myers Company (Syracuse, NY, U.S.A.). The purity of the radioactive compound was at least

99.5% as analysed by reversed-phase HPLC using a Waters μBondapak C_{18} column and methanol-water mixtures in the range of 40-60% methanol as eluents. α-Phenyl-*N*-tert. butylnitrone (PBN) was purchased from Tramedico B.V., Weesp, The Netherlands and α-(4-pyridyl-1-oxide)-N-tert. butylnitrone (POBN) from Janssen Chimica, Beerse, Belgium. Glucose-6-phosphate, glucose-6-phosphate-dehydrogenase, NADP and NADPH were obtained from Boehringer GmbH, Mannheim, F.R.G. Sodium phenobarbital was purchased from Serva Feinbiochemica GmbH, Heidelberg, F.R.G. and ascorbic acid from Gibco Europe B.V., Breda, The Netherlands. SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) was obtained from Smith, Kline and French Laboratories (Philadelphia, PA, U.S.A.). All other chemicals used were reagent grade and were purchased from Merck Nederland B.V., Amsterdam or J.T. Baker Chemicals B.V., Deventer, The Netherlands.

Liver microsomes. Wistar rats (180-200 g) were killed by decapitation. Livers were removed and homogenized at 4°C with a Potter-Elvehjem homogenizer in 1.15% KCl containing 0.01 M phosphate buffer (pH 7.4) (4 ml/g liver). The homogenate was centrifuged for 20 min at 10,000 *g* in an MSE Hi-spin 21 centrifuge and the supernatant was decanted and recentrifuged for 1 hr at 100,000 *g* in an MSE superspeed 65 ultracentrifuge.

Microsomes from Wistar rats pretreated with phenobarbital (1 g/l in drinking water for 10 days) were prepared in the same way [6]. The microsomal pellets were stored at -70°C and used within 1 month of preparation.

Incubation mixtures. Microsomal pellets were resuspended in 0.1 M phosphate buffer (pH 7.4) before incubation. Incubation vessels contained in a final volume of 4 ml: liver microsomal protein, 4 mg; potassium phosphate (pH 7.4), 400 μmol; NADP, 4 μmol; glucose-6-phosphate, 40 μmol; magnesium chloride, 30 μmol; and the substrate [^{3}H]VP 16-213, 68 nmol, being added in 400 μl phosphate buffer.

The reaction was initiated by the addition of 10 μl glucose-6-phosphate-dehydrogenase (0.7 IU/μl). Control vessels contained 0.1 M phosphate buffer (pH 7.4) instead of the NADPH-generating system. Reactions were carried out for 1 hr at 37°C in a GFL shaking incubator and stopped by adding 4 ml saturated $CaCl_2 \cdot 2H_2O$ and 4 ml methanol.

In inhibition experiments the cytochrome P-450 inhibitor SKF-525A and the radical scavenger and reductant ascorbic acid were added to the incubation mixture in 0.1 M phosphate buffer (pH 7.4) at a final concentration of 1 mM, and the spin traps PBN [7] and POBN [8] at final concentrations of 30 and 20 mM, respectively. The inhibitor solutions were prepared immediately before use.

Protein concentration was determined by the Bio-Rad protein assay [9], using bovine serum albumin as the protein standard.

Determination of covalent binding. After the addition of saturated $CaCl_2 \cdot 2H_2O$ and methanol the reaction mixture was centrifuged for 20 min at room temperature at 2500 *g* in a MSE Minor centrifuge. The supernatant was removed and the protein precipitate washed repeatedly with 4 ml methanol-water (4:1, v/v) and 4 ml ethanol-ether (1:1, v/v), until no further radioactivity was found in the extracts (usually 4-5 times with methanol-water and 1-2 times with ethanol-ether). Washing was carried out by stirring for 20 min at 40°C.

The extracted protein pellets were incubated for 1 hr at 50°C with 1 ml soluene-350 and after addition of 15 ml Instagel and 100 μl glacial acetic acid the radioactivity was counted in a Beckman LS 8000 liquid scintillation counter. Radioactivity was corrected for background and for quenching (external standardization).

Aminopyrine N-demethylase activity. Aminopyrine *N*-demethylase activity of rat liver microsomes was determined by McLean and Driver's modification of Nash's method [10, 11]. The effect of VP 16-213 on the kinetics of *N*-demethylation of aminopyrine was determined at the concentration used in the binding experiments: 17 μM.

Covalent binding to HeLa cell microsomal proteins

HeLa CCL2 cells were acquired from Flow Laboratories Ltd (Irvine, Ayrshire, U.K.) and cultured in monolayers in sterile plastic flasks (Costar, Cambridge, U.K., 650 ml) to confluency. The culture medium was a 'Glasgow modification' of Eagle's medium (Flow Laboratories Ltd) containing in a volume of 50 ml: fetal calf serum, 10%; glutamine, 2 mM; non-essential amino acids concentrate (Flow Laboratories Ltd), 1%; streptomycin, 500 μg/ml; and penicillin, 500 IU/ml. Upon removal of the culture medium the HeLa cells were washed twice with 10 ml 50 mM potassium phosphate buffer (pH 7.4) containing 155 mM sodium chloride. The cells were harvested by the addition of 1.8 ml of 50 mM potassium phosphate buffer (pH 7.4) containing EDTA, 0.3 mM; glycerol, 25%; and BSA, 0.04%, and incubated for 2 min. The cell suspension was homogenized consecutively with a polytron mixer (Kinematica, GmbH) and a Potter-Elvehjem homogenizer. The homogenate was centri-

fuged for 5 min at 600 **g** and the supernatant was recentrifuged for 15 min at 8500 **g**, each in an MSE Hi-Spin 21 centrifuge. In order to obtain the microsomal pellet the 8500 **g** supernatant was recentrifuged for 60 min at 100,000 **g** in an MSE Prepspin 75 ultracentrifuge.

Covalent binding of VP 16-213 to HeLa cell microsomal proteins was determined as indicated above for binding to rat liver microsomal proteins with the following modifications: the final incubation volume was 1.3 ml and the buffer used was 0.05 M potassium phosphate (pH 7.4).

Aminopyrine *N*-demethylase activity of HeLa cell microsomes was determined as described above for rat liver microsomes.

Difference spectrophotometry

Binding of VP 16-213 and the spin traps PBN and POBN to ferric cytochrome P-450 was assayed by difference spectrophotometry, using phenobarbital-induced liver microsomal suspensions containing 1.5 mg/ml protein and 1 mM EDTA in 0.05 M potassium phosphate buffer, pH 7.4. The spin traps were added in buffer, VP 16-213 in DMSO. The difference spectra were recorded at 25°C on an Aminco DW 2a™ u.v./vis. double-beam spectrophotometer. The cytochrome P-450 content of the microsomes was determined by the method described by Estabrook *et al.* [12].

Electron spin resonance (ESR) spectroscopy

ESR spectra were recorded at room temperature on a Varian E-3 spectrometer equipped with an E-4531 multipurpose cavity, an aqueous flat cell and a standard quartz tube. The modulation frequency of the spectrometer was 100 kHz.

The incubation mixture contained rat liver microsomal protein (0.5–3.0 mg/ml), NADPH 1 mM, $MgCl_2 \cdot 6H_2O$ 7.5 mM and VP 16-213 1 mM (added in DMSO). Additional incubations were carried out in the presence of the spin traps POBN (20 mM) and PBN (30 mM). For direct ESR measurements using an aqueous flat cell, 1 ml microsomal reaction mixture was incubated at 37°C for 30 sec. For indirect measurements 10 ml microsomal suspension were incubated at 37°C for 10 min and then extracted with 10 ml chloroform. The organic layer was evaporated to dryness under a stream of nitrogen. The residue, dissolved in 0.3 ml N_2-gassed chloroform, was used for ESR investigation.

RESULTS

As will be pointed out in the discussion, oxidative bioactivation of VP 16-213 might involve the following two metabolic routes: radical and/or quinone formation from the phenolic E-ring and carbene formation from the methylenedioxy group. The results of the experiments on covalent binding of ^{3}H-labeled VP 16-213 to rat liver microsomal proteins are shown in Table 1. After incubation of [^{3}H]VP 16-213 with rat liver microsomes for 1 hr, 6% of the incubated radioactivity was found to be covalently bound to the microsomal proteins. In the absence of NADPH covalent binding decreased to 15% of the control value. Addition of PBN, ascorbic acid, SKF-525A or POBN to the incubation mixture caused a decrease in covalent binding to 16, 17, 46 and 85%, respectively. Binding to microsomes from phenobarbital-treated rats amounted to 131% of the control value.

Table 1. *Effects of incubation conditions on* in vitro *covalent binding of* [^{3}*H*]*VP* 16-213 *to rat liver microsomes*•

Addition	Efficiency of binding (%)†	% of radioactivity covalently bound to control microsomes‡
NADPH	6.1 ± 0.3	100 ± 5
—	0.9 ± 0.2	15 ± 3§
NADPH + SKF-525A	2.8 ± 0.3	46 ± 5
NADPH + ascorbic acid	1.0 ± 0.1	17 ± 2
NADPH + PBN	1.0 ± 0.2	16 ± 4
NADPH + POBN	5.2 ± 0.2	85 ± 4
PB‖ + NADPH	8.0 ± 0.4	131 ± 6
PB	1.0 ± 0.2	17 ± 3
PB + NADPH + SKF-525A	3.2 ± 0.3	53 ± 5

•Mean ± S.D. for at least four experiments. The amount of radioactivity added to the incubation mixtures was (52.3 ± 0.5) × 10^5 dpm.

†% of the radioactivity which was covalently bound.

‡The average amount of radioactivity covalently bound to control microsomes was (3.2 ± 0.2) × 10^5 dpm.

§$P < 0.001$ for all values when compared to control.

‖Microsomes from rats pretreated with phenobarbital.

Omittance of NADPH and addition of SKF-525A decreased covalent binding to phenobarbital-induced microsomes to respectively 17 and 53% of the control value. If [^{3}H]VP 16-213 was incubated with aliquots of the 100,000 *g* supernatant of a rat liver homogenate, no covalent binding was observed.

In order to study the mechanism of the above decreasing effect of the well-known radical scavengers PBN and POBN on covalent binding we studied their interaction with cytochrome P-450 using difference spectrophotometry. The interaction of the spin traps PBN and POBN with VP 16-213 for binding to cytochrome P-450 was also investigated. VP 16-213 produced a reverse type I difference spectrum (Fig. 2A). A considerable difference was found between the two spin traps PBN and POBN in affecting the spectrum of VP 16-213. POBN had almost no effect (spectrum not shown), whereas PBN markedly reduced the difference spectrum of VP 16-213 (Fig. 2B). The spectrum of VP 16-213 in the presence of PBN can be interpreted as the sum of a weak VP 16-213 spectrum and a reverse type I PBN spectrum both from the sample cell, and a stronger PBN spectrum from the reference cell. PBN has been reported to be a type I substrate if concentrations up to 2 mM are used [13]. In this study, however, a reverse type I spectral change was evoked if concentrations higher than 10 mM were used (Fig. 2C). The spin trap POBN had little effect on the difference spectrum of VP 16-213. In fact, the absorbance difference (ΔA) of the spectrum of VP 16-213 decreased in the same order of magnitude

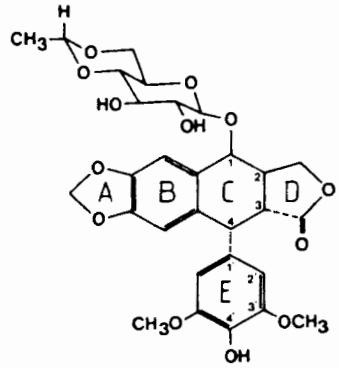

Fig. 1. Structure of VP 16-213.

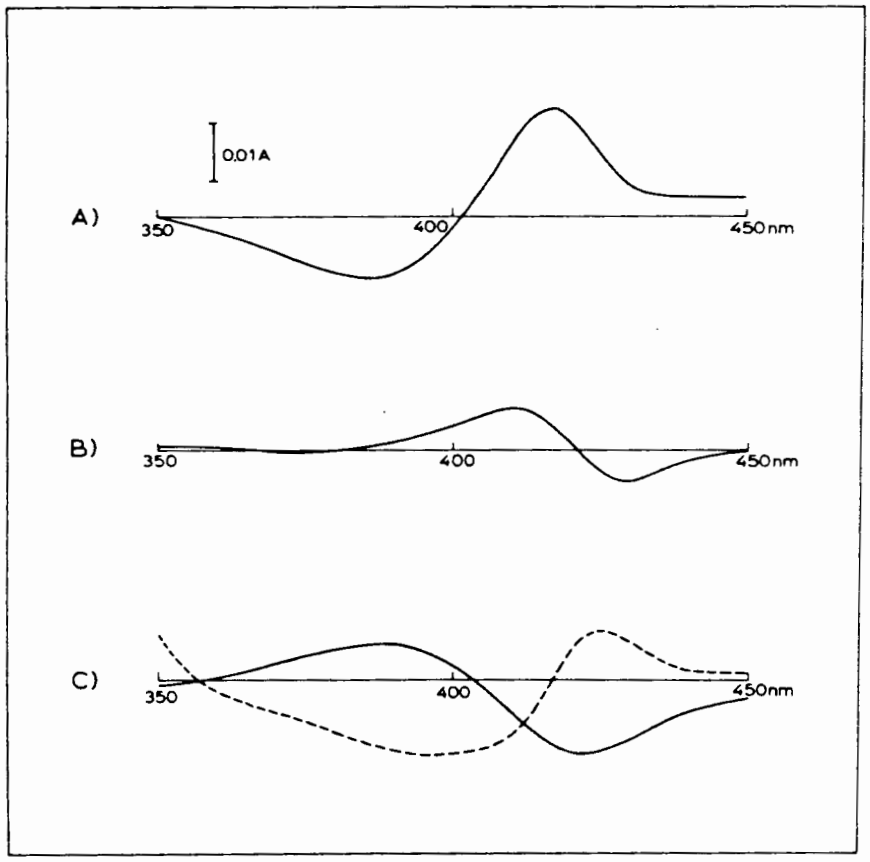

Fig. 2. Binding of VP 16-213 to cytochrome P-450 and the influence of PBN. Difference spectra: (A) reference cell: microsomal suspension (MIC), sample cell: MIC + VP 16-213 (17 μM); (B) reference cell: MIC + PBN (30 mM), sample cell: MIC + PBN (30 mM) + VP 16-213 (17 μM); (C) reference cell: MIC, sample cell: MIC + PBN (3 mM) (——), MIC + PBN (30 mM) (- - -).

as the covalent binding of the drug to microsomal proteins on addition of POBN: 20 and 15%, respectively.

The possibility of carbene formation was investigated by studying the effect of VP 16-213 on the kinetics of rat hepatic aminopyrine *N*-demethylation. From the plots shown in Fig. 3, the following V_{max} and $1/K_m$ values were calculated: in the absence of VP 16-213: $1/K_m = 2.80 \pm 0.33$ and $V_{max} = 179 \pm 22$; in the presence of VP 16-213: $1/K_m = 2.47 \pm 0.24$ and $V_{max} = 176 \pm 20$. These values show that addition of VP 16-213 to the incubation mixture had no inhibitory effect on aminopyrine *N*-demethylase ($0.10 < P < 0.20$ for $1/K_m$ when compared to control; each $1/K_m$ value is the mean and S.D. of four assays).

Led by the suggestion of Loike and Horwitz that formation of a chemically reactive metabolite accounts for the induction of breaks in HeLa cell DNA [2], we looked for the presence of cytochrome P-450 in HeLa cells. The presence of cytochrome P-450 in HeLa cells was investigated by an attempt to determine aminopyrine *N*-demethylase activity in HeLa cell microsomes. The activity was found to be 390 ± 20 pmol H_2CO/min/mg microsomal protein ($n = 4$), which is about eight times lower than that in rat liver microsomes (3200 ± 100 pmol H_2CO/min/mg microsomal protein, $n = 4$). Aminopyrine *N*-demethylase activity of HeLa cell microsomes decreased from 390 pmol H_2CO/min/mg protein at 5 min to 260 pmol H_2CO/min/mg protein at 20 min. Covalent binding of ^{3}H-labeled VP 16-213 was also studied using HeLa cell microsomes. Covalent binding of radiolabel to HeLa cell microsomal proteins was mainly dependent on NADPH (see Table 2). The binding to HeLa cell microsomes was found to be less extensive then that to rat liver microsomes.

DISCUSSION

The molecular structure of VP 16-213 has two main components which might be involved in its oxidative biotransformation: (1) the phenolic ring and (2) the methylenedioxy group.

(1) The sterically hindered phenolic structure (ring E) is related to that of the widely used antioxidant 3,5-ditert. butyl-4-hydroxytoluene (BHT), the metabolism of which has been the subject of a number of investigations. It has been proposed that one of the cytochrome P-450-mediated metabolic routes of BHT in the rat and man is free radical formation [14], possibly resulting in quinone formation [15]. In the case of VP 16-213, a similar route of biotransformation should be considered.

(2) the methylenedioxy group (ring A) of several compounds, such as piperonylbutoxide, has been reported to be oxidized to a carbinol by cytochrome P-450 followed by dehydration to a carbene [16]. This carbene forms a complex with the heme iron in cytochrome P-450, resulting in inhibition of cytochrome P-450. In this respect it is of interest that podophyllotoxin, the parent compound of VP 16-213, has been shown to be a moderate inhibitor of cytochrome P-450 [17].

In an earlier study [18] we observed that in incubations of ^{3}H-labeled VP 16-213 with rat liver microsomes 5–10% of incubated radioactivity was recovered in the microsomal pellet. We now studied in detail the possible covalent binding of ^{3}H-labeled VP 16-213 to rat liver microsomal proteins and the relation of this process to the two previously mentioned possible metabolic routes of VP 16-213. After incubation of [^{3}H]VP 16-213 with rat liver microsomes for 1 hr, 6% of the incubated radioactivity was found to be covalently bound to the microsomal proteins. Furthermore, the effects of incubation conditions indicate that covalent binding is NADPH-

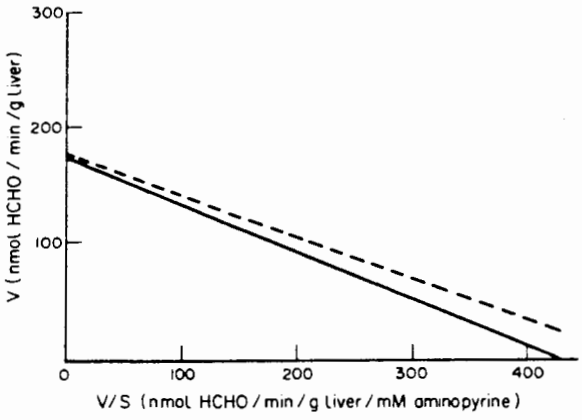

Fig. 3. Eady-Hofstee plots of N-demethylation of aminopyrine by rat liver microsomes in the absence (——) and presence (- - -) of 17 μM VP 16-213.

*Table 2. Covalent binding of [^{3}H]-VP 16-213 to HeLa cell microsomes**

Addition	Efficiency of binding (%)†	% of radioactivity covalently bound to control microsomes‡
NADPH	4.0 ± 0.2	100 ± 4
—	1.4 ± 0.1	36 ± 3§

*Mean ± S.D. for three experiments. The amount of radioactivity added to the incubation mixtures was $(17.0 \pm 0.2) \times 10^5$ dpm.

†% of the radioactivity which was covalently bound.

‡The average amount of radioactivity covalently bound to control microsomes was $(0.68 \pm 0.03) \times 10^5$ dpm.

§$P < 0.001$ compared to control.

dependent. The background binding of 15% in the absence of NADPH could be due to the fact that VP 16-213 is not completely removed in the washing procedure. Phenobarbital treatment increased hepatic covalent binding, whereas addition of SKF-525A, a well-known inhibitor of the microsomal cytochrome P-450-linked monooxygenase system, resulted in a statistically significant decrease of covalent binding to normal as well as phenobarbital induced microsomes. No binding was observed to the 100,000 *g* supernatant of a rat liver homogenate. These results indicate that cytochrome P-450-mediated oxidative metabolic activation plays a role in covalent binding of VP 16-213 to rat liver microsomal proteins.

The first manner in which cytochrome P-450-mediated activation could play a role in covalent binding, viz. by oxidation of the ring structurally related to BHT, was studied because the latter compound is known to be activated by cytochrome P-450 to reactive intermediate(s) capable of being covalently bound to hepatic microsomal proteins [19]. Addition of ascorbic acid, a well-known free radical scavenger as well as reducing agent, decreased the covalent binding of VP 16-213 to the same low level as in the case of omission of NADPH. This suggests that, as in the case of BHT, radical [14] and/or quinone formation [15] by mixed-function oxidase from the phenolic structure of VP 16-213 may be involved in the binding process. We investigated the possibility of radical formation by examining the effects of the spin traps PBN and POBN on covalent binding. The concentrations of the spin traps used (30 mM for PBN and 20 mM for POBN) were based on literature data of efficiency [13]. PBN appeared to be much more efficient in inhibiting covalent binding of VP 16-213 intermediates than POBN, and decreased binding to the same level as if NADPH was omitted. However, incubations of VP 16-213 with rat liver microsomes + NADPH in the presence or absence of the spin traps PBN and POBN did not give rise to a detectable ESR signal, measured either directly or indirectly after extraction with chloroform. It should be noted that we [20] as well as others [21] have observed formation of the VP 16-213 phenoxy radical by horseradish peroxidase and myeloperoxidase in the presence of hydrogen peroxide. The phenoxy radical generated by these systems is detectable in the presence of 1 mg/ml microsomal protein, indicating that the failure to detect the phenoxy radical in microsomal incubations is not due to scavenging by microsomal proteins. However, the sensitivity of the method of detection may not be sufficiently high.

Since PBN and POBN have been reported to inhibit cytochrome P-450 [13], we investigated their interaction with VP 16-213 for binding to the hemoprotein using difference spectrophotometry. PBN markedly reduced the difference spectrum of VP 16-213, while POBN had almost no effect. From the ESR and difference spectrophotometry experiments we conclude that (1) VP 16-213 is able to bind to cytochrome P-450; (2) no free radical is detected in incubations of VP 16-213 with rat liver microsomes + NADPH; and (3) the difference in inhibition of covalent binding to microsomal proteins between the spin traps PBN and POBN is due to a difference in inhibition of cytochrome P-450 between these compounds. The effects of the spin traps give further support to the assumption that activation of VP 16-213 by cytochrome P-450 to a binding intermediate occurs.

The second way in which cytochrome P-450-mediated activation could play a role in covalent binding, the oxidation of the methylenedioxy group, was investigated by studying the effect of VP 16-213 on the activity of rat hepatic aminopyrine *N*-demethylase. When oxidation of the methylenedioxy group occurs, resulting in inhibition of cytochrome P-450 (see above), the activity of aminopyrine *N*-demethylase should be reduced. However, at the concentration used in the binding experiments (17 μM), VP 16-213 had no effect on the aminopyrine *N*-demethylase activity. This suggests that metabolism of the methylenedioxy group does not play a role in metabolic activation of VP 16-213 to intermediates binding to microsomal proteins.

It is of interest that evidence for the presence of cytochrome P-450 in HeLa cells was obtained by the observation that aminopyrine underwent *N*-demethylation when incubated with HeLa cell microsomes. The activity decreased in time, indicating marked instability of cytochrome P-450 in the HeLa cell, which is possibly due to proteolytic activity in HeLa cells.

Because of the finding of mixed-function oxidase activity in HeLa cells, covalent binding was also studied using HeLa cell microsomes. Covalent binding of radiolabel to HeLa cell microsomal proteins was mainly dependent on NADPH. The observation that covalent binding to HeLa cell microsomes was less extensive then that to rat liver microsomes might be explained by the fact that HeLa cells have a low metabolic capacity. These findings indicate that covalent binding of VP 16-213 to HeLa cell microsomes results from conversion to a chemically reactive species and that cytochrome P-450 is involved in this process.

In conclusion the present work demonstrates that transformation of VP 16-213 by the phenobarbital-inducible cytochrome P-450 isoenzyme may play a role in the covalent binding of

VP 16-213 to rat hepatic and HeLa cell microsomal proteins. Indirect evidence was obtained for the involvement of the phenolic function of the VP 16-213 structure in the binding process. Further studies to investigate the mechanism of the oxidative activation of VP 16-213, e.g. the possibility of quinone formation, are ongoing.

REFERENCES

1. D'Incalci M, Garattini S. Podophyllotoxin derivatives VP-16 and VM-26. In: Pinedo HM, Chabner BA eds. *The EORTC Cancer Chemotherapy Annual.* Amsterdam, Elsevier, 1983, Vol. 5, 100-106.
2. Loike JD, Horwitz SB. Effect of VP 16-213 on the intracellular degradation of DNA in HeLa cells. *Biochemistry* 1976, **15**, 5443-5448.
3. Wozniak AJ, Ross WE. DNA damage as a basis for 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside) (Etoposide) cytotoxicity. *Cancer Res* 1983, **43**, 120-124.
4. Gillette JR. A perspective on the role of chemically reactive metabolites of foreign compounds in toxicity: I. Correlation of changes in covalent binding of reactive metabolites with changes in the incidence and severity of toxicity. *Biochem Pharmacol* 1974, **23**, 2785-2794.
5. Gillette JR. A perspective on the role of chemically reactive metabolites of foreign compounds in toxicity: II. Alteration in the kinetics of covalent binding. *Biochem Pharmacol* 1974, **23**, 2927-2938.
6. Foster AB, Jarman M, Kinas RW *et al.* 5-Fluoro- and 5-chlorocyclophosphamide: synthesis, metabolism, and antitumor activity of the *cis* and *trans* isomers. *J Med Chem* 1981, **24**, 1399-1403.
7. Pyer JL, Floyd RA, McCay PB, Janzen EG, Davis ER. Spin-trapping of the trichloromethyl radical produced during enzymatic NADPH oxidation in the presence of carbon tetrachloride or bromotrichloromethane. *Biochem Biophys Acta* 1978, **539**, 402-409.
8. Janzen EG, Wang YY, Shetty RV. Spin-trapping with α-pyridyl-1-oxide *N*-tert-butyl nitrones in aqueous solutions. A unique electron spin resonance spectrum for the hydroxyl radical adduct. *J Am Chem Soc* 1978, **100**, 2923-2925.
9. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, **72**, 248-254.
10. McLean AM, Driver DE. Combined effects of low doses of DDT and phenobarbital on cytochrome P-450 and aminopyrine demethylation. *Biochem Pharmacol* 1977, **26**, 1299-1302.
11. Nash T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* 1953, **55**, 416-421.
12. Estabrook RW, Peterson JA, Baron J, Hildebrandt A. Spectrophotometric measurement of turbid suspensions of cytochromes associated in drug metabolism. In: Chignell CF, ed. *Methods in Pharmacology.* New York, Appleton-Century-Crofts, 1972, Vol. 2, 303-350.
13. Augusto O, Beilan HS, Ortiz de Montellano PR. The catalytic mechanism of cytochrome P-450. Spin-trapping evidence for one-electron substrate oxidation. *J Biol Chem* 1982, **257**, 11288-11295.
14. Daniel JW, Gage JC, Jones DI. The metabolism of 3,5-di-tert-butyl-4-hydroxytoluene in the rat and in man. *Biochem J* 1968, **106**, 783-790.
15. Nakagawa Y, Hiraga K, Suga T. On the mechanism of covalent binding of butylated hydroxytoluene to microsomal protein. *Biochem Pharmacol* 1983, **32**, 1417-1421.
16. Hodgson E. In: Hodgson E, Guthrie FE eds. *Introduction to Biochemical Toxicology.* Amsterdam, Elsevier, 1980, 151.
17. Fujii K, Jaffe H, Bishop Y, Arnold E, Mackintosh D, Epstein SS. Structure-activity relations for methylenedioxyphenyl and related compounds on hepatic microsomal enzyme function, as measured by prolongation of hexobarbital narcosis and zoxazolamine paralysis in mice. *Toxicol Appl Pharmacol* 1970, **16**, 482-494.
18. van Maanen JMS, van Oort WJ, Pinedo HM. *In vitro* and *in vivo* metabolism of VP 16-213 in the rat. *Eur J Cancer Clin Oncol* 1982, **18**, 885-890.
19. Nakagawa Y, Hiraga K, Suga T. Biological fate of butylated hydroxytoluene (BHT): binding *in vitro* of BHT to liver microsomes. *Chem Pharm Bull (Tokyo)* 1979, **27**, 480-485.

20. van Maanen JMS, de Ruiter C, Kootstra PR, de Vries J, Pinedo HM. Free radical formation of VP 16-213. *Proc AACR* 1984, **25**, 384.
21. Sinha BK, Trush MA, Kalyanaraman B. Free radical metabolism of VP-16 and inhibition of anthracycline-induced lipid peroxidation. *Biochem Pharmacol* 1983, **32**, 3495–3498.