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IRREVERSIBLE BINDING OF ETOPOSIDE (VP-16-213) TO DEOXYRIBONUCLEIC ACID AND PROTEINS

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VP-16-213 (NSC 141540, Fig. 1), a semi-synthetic podophyllotoxin derivative, is an important drug for the treatment of small cell carcinoma (1). Loike and Horwitz (2) and, more recently, Wozniak and Ross (3) and Long et al. (4) have shown that VP-16 induces DNA damage in L1210 and human lung adenocarcinoma cells and have suggested that the cytotoxicity of VP-16 may be due to this DNA damage. Furthermore, a free hydroxyl group at the 4'-position was essential for cytotoxicity. We have shown recently that VP-16 forms an oxygen-centered free radical intermediate when activated by horseradish peroxidase in the presence of H_2O_2 (5). While these events may be important for the cytotoxicity of VP-16, the cellular and molecular mechanism(s) of action of VP-16 remains obscure.

We have recently shown that VP-16 undergoes O-demethylation in the presence of mouse liver microsomes and NADPH which may result in the formation of reactive intermediates (6). In this communication, we show that the reactive intermediate(s) formed during microsomal activation of VP-16 binds irreversibly to nucleic acid and microsomal proteins.

MATERIALS AND METHODS

VP-16-213 (NSC 141540) was obtained from the Drug Development Branch, National Cancer Institute, NIH, Bethesda, MD. [^{3}H]-VP-16 (sp. act. 200 mCi/mmole; >97% radiochemical purity by HPLC, C_{18} reverse phase, methanol : H_2O 3:2) was purchased from Moravek Biochemicals, Brea, CA. Calf thymus DNA (highly polymerized, type I), NADPH, NADH, phenobarbital sodium salt (PB), and polyethylene glycol (mol. wt ~200) were obtained from the Sigma Chemical Co., St. Louis, MO.

Mouse liver microsomes and PB-induced (80 mg/kg; i.p. x 3 daily) microsomes were prepared from CDF male mice according to a published method (7). Food and water were provided ad lib. The protein concentration of the microsomes was determined according to the method of Sutherland et al. (8) using bovine serum albumin as a standard.

Nucleic acid was dissolved in 150 mM KCl-50 mM Tris-HCl buffer (pH 7.4) at a concentration of 1 mg/ml, and VP-16 (500μM containing 1μCi/ml [^{3}H] VP-16) dissolved in polyethylene glycol (final concentration ~0.5%) was added in a total volume of 5 ml. After mixing for 15 min at room temperature, microsomes (final concentration = 1 mg/ml) were added and the mixture was incubated at 37° for 2 min with shaking. The reaction was initiated by adding either NADPH (2mM) or NADH (2 mM) and was incubated for an additional 60 min at 37°. The reaction

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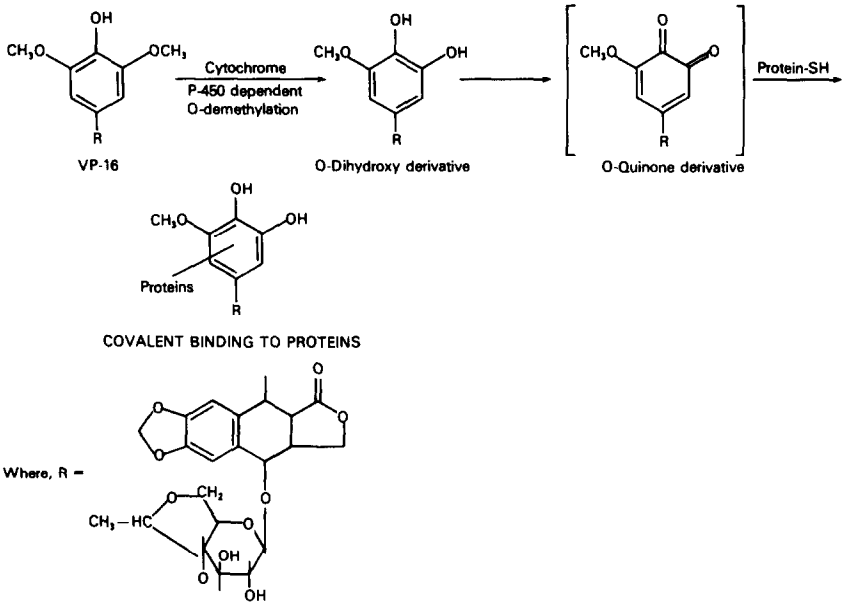

Fig. 1. Structure and proposed activation of VP-16 to reactive intermediates and covalent binding to cellular macromolecules.

mixtures were centrifuged (4°, 15,000 g, 20 min), and the clear supernatant fraction containing DNA was removed and extracted with equal volumes of water saturated chloroform-phenol (1:1) as described previously (9,10). The mixture was centrifuged (6,000 g, 20 min), the clear supernatant fraction was removed, and the DNA was precipitated in 4 vol. of cold ethanol. The ethanol precipitate was collected, washed with ethanol (3 x 25 ml), dried with nitrogen, dissolved in the buffer, and reprecipitated with ethanol. The precipitated DNA-drug complexes were collected, washed with ethanol (3 x 25 ml), dried, and redissolved in the 2 ml of the buffer. Under these conditions of isolation, most of the unbound radioactivity was removed from the controls. The irreversibly bound VP-16 metabolite in the sample was measured with a Beckman scintillation counter (LS 9000) using aqueous counting scintillant (Amersham Corp., Arlington Heights, IL) and the appropriate quenching corrections using an external standard were made. The nucleotide concentration in the samples was determined as described previously (11). The binding ratio is the molar ratio of drug to mononucleotide unit.

The irreversibly bound VP-16 metabolite(s) to the microsomal proteins was isolated by washing the pelleted microsomes with buffer (3 x 15 ml) to remove traces of DNA, and then with methanol (3 x 15 ml). The microsomes were dissolved in 1% sodium dodecyl sulfate (SDS), and precipitated with 20% trichloroacetic acid (TCA). The precipitated protein was washed with 5% TCA (3 x 15 ml), buffer (3 x 15 ml), methanol (3 x 15 ml), and dissolved in 0.5 M NaOH; the bound drug was quantitated in the samples.

RESULTS AND DISCUSSION

Table 1 shows that incubation of VP-16 with DNA in the presence of heat-denatured microsomes resulted in some binding, representing 1 mole of drug bound per 17,000 base pair. More drug was bound to DNA in the presence of native microsomes. In the presence of NADPH, however, a significant increase in the binding of VP-16 metabolite(s) to DNA was observed (Table 1). The presence of PB-induced microsomes and NADPH also stimulated the binding. NADH, in contrast to NADPH, was without any effect on the binding, and the binding ratios were similar to that obtained with the native microsome alone.

Table 1. Binding of [^{3}H]VP-16-213 to DNA and Microsomal Proteins

Conditions*	DNA (binding ratio x 10^4)	Proteins (nmoles/g)
Heated microsomes# + NADPH	0.58 ± 0.2	25 ± 6
Microsomes	1.2 ± 0.25	28 ± 8
Microsomes + NADPH	2.9 ± 0.25$^+$	91 ± 16$^+$
Microsomes + NADH	1.16 ± 0.17	43 ± 5
PB-induced microsomes + NADPH	8.5 ± 0.1§	190 ± 10§

*VP-16 (500 μM) was incubated with DNA (1 mg/ml) and mouse liver microsomes (1 mg/ml) in the presence or absence of cofactors for 60 min at 37° (see Materials and Methods for details). The binding ratio is the molar ratio of drug to mononucleotide unit. Values represent the average of four determinations.
#Microsomes were heated at 80° for 20 min.
$^+$Significantly different ($p<0.05$) from microsomes alone.
§Significantly different ($p<0.01$) from microsomes + NADPH.

Similarly, a small amount of the drug became bound to either the heat-denatured or the native microsomal protein. NADPH increased this binding significantly (Table 1). The presence of PB-induced microsomes and NADPH further increased the binding of VP-16 metabolites to proteins. Again, NADH did not stimulate the binding of the drug.

The present study clearly demonstrates that an active intermediate derived from VP-16 bound irreversibly to both DNA and microsomal proteins. The binding was enzymatic and NADPH dependent. Moreover, the formation of active intermediate was increased in the presence of PB-induced microsomes. Whether a free radical intermediate (5) or some species derived from this free radical intermediate irreversibly binds to DNA and protein is not known. Recently, we have shown that VP-16 undergoes O-demethylation in the presence of mouse liver microsomes and NADPH which is enhanced by PB-induced microsomes (6). This O-demethylation of VP-16 may generate an active species, an o-quinone derivative, which may bind covalently to proteins and other cellular macromolecules, as shown in Fig. 1.

The exact relationship between covalent binding of VP-16 metabolite(s) to cellular macromolecules and cytotoxicity is not known at this time. However, such binding may play a major role in inhibiting DNA template function and protein synthesis resulting in cell death.

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