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# PEROXIDATIVE FREE RADICAL FORMATION AND O-DEMETHYLATION OF ETOPOSIDE(VP-16) AND TENIPOSIDE(VM-26)

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The peroxidative activation of the antitumor drugs, etoposide(VP-16) and teniposide (VM-26), has been studied in vitro. Both of these drugs, in the presence of horseradish peroxidase or prostaglandin synthetase, formed phenoxy radical intermediates. Furthermore, this activation also resulted in the formation of two metabolites from each of the drugs. Using HPLC and mass spectrometry, one of the metabolites was shown to be the reactive o-quinone derivative of the parent drug which resulted from the peroxidative O-demethylation. It appears that O-demethylation catalyzed by peroxidases may be an important mechanism for the formation of reactive intermediates and may play a role in the mechanism of action of VP-16 and VM-26. © 1986 Academic Press, Inc.

The semisynthetic podophyllotoxin derivatives,VP-16 and VM-26, are clinically active antitumor drugs(1). Although their mechanism of action is not clear, recent studies have shown that both VP-16 and VM-26 induce single- and double-strand DNA breaks in tumor cells(2-6). Cellular activation is required for this DNA damage(2). Studies from our laboratories have shown that VP-16 undergoes O-demethylation catalyzed by liver microsomes in the presence cf NADPH, and forms reactive intermediates, which bind irreversibly to microsomal proteins or to exogenously added DNA(7,8). We have previously proposed that microsomal activation of VP-16 is cytochrome P-450-dependent and that the o-quinone derivative of VP-16 may be the alkylating species. Recently, peroxidases have been shown to be important cellular enzymes responsible for the one-electron activation of drugs and carcinogens(9-12). Peroxidase catalyzes the O-demethylation of ellipticine derivative(13), and VP-16 forms a stable oxygen-centered radical during horseradish peroxidase(HRP)activation(14). We have, therefore, examined the ability of HRP and prostaglandin synthetase(PGS) to catalyze the O-demethylation

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of VP-16 and VM-26. In this report, we show that both VP-16 and VM-26 undergo O-demethylation in the presence of either PGS or HRP, and that their phenoxy free radicals are the pivotal intermediates for the direct formation of the highly reactive o-quinone derivatives.

## MATERIALS AND METHODS

VP-16 and VM-26 were gifts from Bristol-Myers Pharmaceuticals, Syracuse, N.Y. The O-quinone derivatives of VP-16 and of VM-26 were synthesized. Standards were dissolved in methanol and stored at -70°c. HRP(type VI) and indomethacin were obtained from Sigma Company, St. Louis, MO., and purified PGS (Prostaglandin H Synthase, prepared from ram vesicle microsomes) was obtained from Oxford Biomedical Research Inc. Oxford M.I. Arachidonic acid (AA,>99% pure)was obtained from Nu-Chek-Prep, Inc., Elysian, Minnesotta.

Reactions for the HPLC analysis were carried out in 150mM KCl and 50mM Tris.HCl buffer pH 7.4 in a total volume of 1ml. The enzyme(HRP 1mg or purified PGS 7500 units) and the substrate (VP-16 250 $\mu$ M dissolved in polyethylene glycol or VM-26, 250 $\mu$ M, dissolved in dimethylsulfoxide, final volume of the vehicles-0.25%) were added to the reaction mixtures on ice. The mixture was warmed to 37°c for 2 min, and the reaction was initiated by adding either $H_2O_2$ (500 $\mu$ M) for the HRP or AA (400 $\mu$ M,dissolved in ethanol) for the PGS reactions. When inhibition by indomethacin was studied, the drug (400 $\mu$ M dissolved in acetone)was preincubated with the enzyme on ice for 5min before adding AA. The reactions were terminated by adding 5ml of chloroform.

The extraction of the incubation mixture was carried out by shaking for 15 min with 5 ml of chloroform. Following centrifugation the organic layer was separated, and the aqueous layer was reextracted with 3 ml of chloroform. The organic layers(8 ml) were collected and evaporated under a nitrogen stream at room temperature. The residue was dissolved in methanol and kept at -70°c until analysis.

The HPLC analysis for the presence of metabolites was carried out with methanol and water(60:40, V/V)as the mobile phase. The solvent was pumped isocratically at 1 ml/min., through a Bondapak phenyl column with the detector set at 277 nm.

The mass spectrometric analysis was carried out on a VG Micromass ZAB-2F mass spectrometer(VG Instrument, Manchester, England) operating in EI ionization and double focusing mode. The samples, dissolved in methanol-water, were allowed to evaporate on the probe wire and introduced into the source. The spectra presented have been background subtracted via a nominal mass background subtraction routine using the mass spectrometer data system.

The electron spin resonance(ESR) spectra were recorded on a Varian E-104 at room temperature(22°C). VP-16 or VM-26(1mM, dissolved in polyethylene glycol) and enzyme (HRP 1 mg/ml or PGS 3500 U/ml) were added in total volume of 1 ml of phoaphate-saline buffer(pH 7.4). The reaction was initiated by adding either $H_2O_2$ or AA(500 $\mu$ M) and the resulting spectrum was recorded. When added, indomethacin(400 $\mu$ M) was preincubated with PGS for 5 min on ice before adding AA.

## RESULTS AND DISCUSSION

Because HRP/$H_2O_2$ activates VP-16 to its phenoxy radical intermediate, we have investigated the potential activation of the VP-16 and VM-26 to radical intermediates using a PGS/AA system. The incubation of either VP-16 or VM-26 with purified PGS in the presence of AA resulted in the formation of an oxygen-centered free radical intermediate. The multi-line ESR spectrum was identical to that obtained with HRP/$H_2O_2$ with either VP-16( data not shown)or VM-26(Figure-1A,1B). Indomethacin, an inhibitor

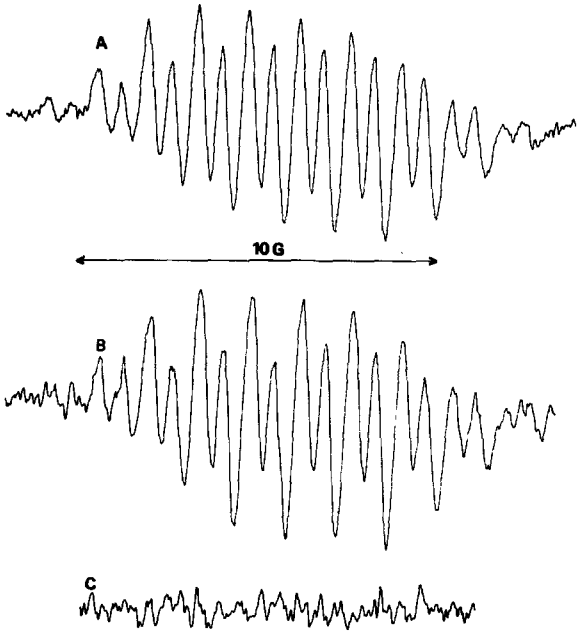



Figure-1. The electron spin resonance spectrum obtained from (A) VM-26(1mM) during incubation with horseradish peroxidase(1 mg/ml) in the presence of $H_2O_2$(500 μ M) at pH 7.4 in phosphate-saline buffer, (B) the spectrum obtained from VM-26(1mM) during the incubation with prostaglandine synthetase(3500 units/ml) and AA(500 μ M) and and (C) same as (B) except it contained indomethacin(400 μ M).
The ESR settings were: field = 3395 G; field scan 20 G; modulation amplitude = 0.5 G; nominal microwave power = 20 G; and the receiver gain was $2.5 \times 10^4$ for (A); $8 \times 10^4$ for (B) and $1.25 \times 10^4$ for (C).

of cyclooxygenase(15), completely inhibited the formation of the radicals(Figure-1C). This would suggest that the dioxygenation of AA to the cyclic hydroperoxyendoperoxide, $PGG_2$, is required. Furthermore, under anaerobic conditions in the presence of $H_2O_2$, an identical ESR spectrum was obtained(data not shown) suggesting that the peroxidase component of the PGS can also activates VP-16 and VM-26, through one-electron oxidation, to the respective radical intermediates during the reduction of $PGG_2$ to $PGH_2$. Because peroxidases are known to carry out demethylation reactions similar to cytochrome P-450, we examined the O-demethylation of VP-16 and VM-26 and the formation of their o-quinone derivatives by HRP and PGS systems. When either VP-16 or VM-26 was incubated with peroxidases(HRP/$H_2O_2$ or PGS/AA) and the chloroform extracts subjected to HPLC analysis, two metabolites, one with an identical retention time of the quinone derivatives of the parent drug and one with a longer retention time than the parent drug, were detected (Figure-2). The formation of these metabolites required all the three components: the

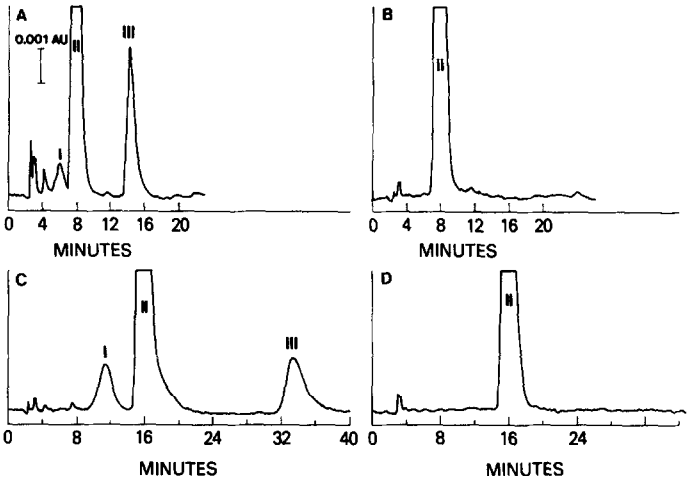



Figure-2. HPLC chromatograms of the chloroform extracts of incubations containing (A) VP-16(250 μ M) and prostaglandin synthetase(7500 units/ml) in the presence of AA(400 μ M); (B) identical to (A) except AA was omitted ;(C) VM-26(250 μ M) in the presence of horseradish peroxidase(1 mg/ml) and $H_2O_2$(500 μ M) and(D) identical to (C) except $H_2O_2$ was omitted. I, II and III in the chromatogramm represent the o-quinone, the parent drug and the unknown metabolite, respectively. The mobile phase for the HPLC analysis was methanol-water(60:40 ; V/V). All incubations were carreied out at 37°C for 15 min.

enzymes, the drugs and either AA or $H_2O_2$. When indomethacin(400 μ M) was included in the incubation mixtures containing PGS and AA, metabolites were not detected.

The identity of one of the metabolites was confirmed by mass spectrometry. Figure-3 compares mass spectra of the authentic quinone derivative of the drugs to the spectra of the metabolite isolated by HPLC with retention times identical to that of the standard quinone. The spectra of the authentic quinone of VP-16 and the metabolite are quite similar and contained the same molecular ion peak(M/Z = 574) and the base peak at 366, which results from the loss of the sugar moiety. In addition, there are a number of other common fragment ions e.g. 337, 251, 221 etc. are also present. The mass spectra of VM-26 quinone and the correspnding metabolite were also similar and contained the same base peak at 366(data not shown). Identical retention times and similar mass spectra confirm that the o-quinone derivatives of VP-16 and VM-26 are formed as a consequence of HRP or PGS activation. The identity of the second metabolite is unknown at this time, however, it is currently under investigation.

The present study indicates that both VP-16 and VM-26 form oxygen-centered phenoxy free radicals during HRP/$H_2O_2$ and PGS/AA activation and that this peroxidative activation results in the formation of two metabolites. Using HPLC and mass spectro-

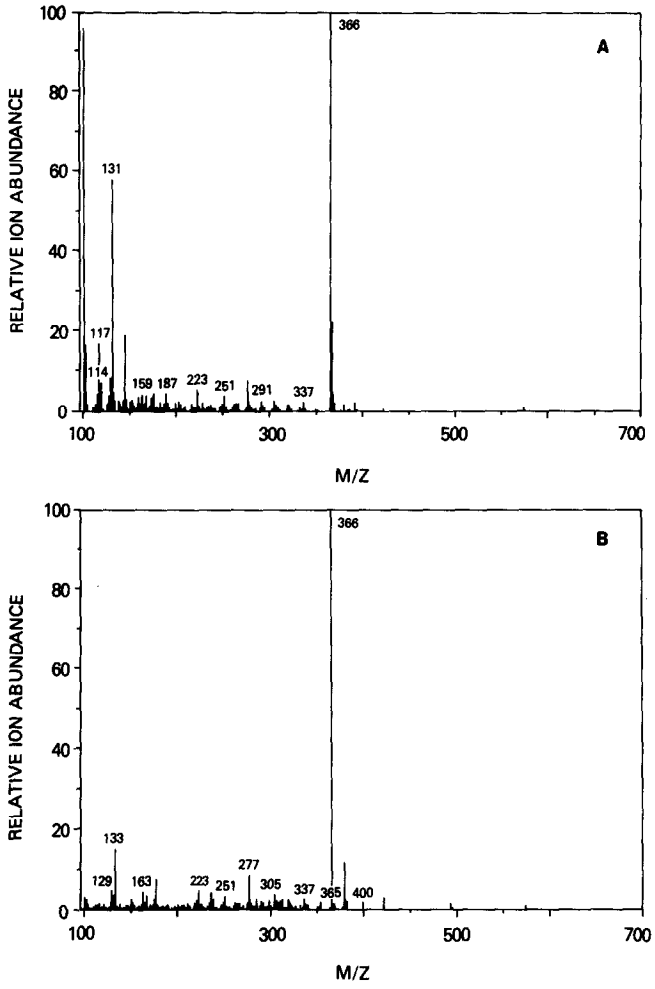
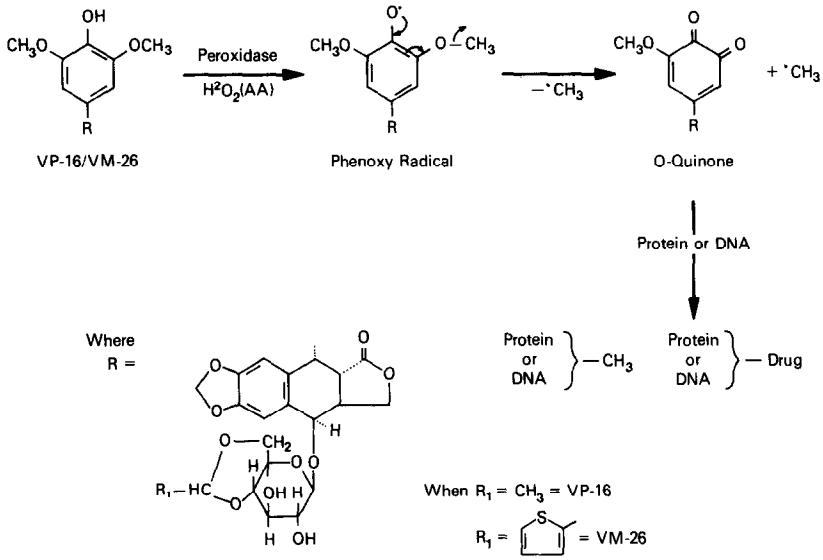



Figure-3. The electron impact mass spectrum obtained from (A) authentic VP-16-quinone (B) the metabolite with identical retention time with VP-16-quinone obtained during horseradish peroxidase and $H_2O_2$ incubation. The metabolite were extracted from the incubation mixtures, collected with HPLC, and reextracted with chloroform.

metric analysis, we have identified one metabolite as the o-quinone derivative of of the parent drug which results from the peroxidase-mediated O-demethylation. We propose that the phenoxy radical is an obligatory intermediate for the formation the o-quinones(Scheme-1). In this scheme, formation of a very reactive $\cdot CH_3$, which is is known to alkylate cellular DNA and proteins, is also postulated. Peroxidase-catalyzed O-demethylation of VP-16 and VM-26 thus appears to be a novel metabolic pathway for the formation of multiple reactive intermediates,($\cdot CH_3$ and drug o-quinones), which could bind to critically important cellular macromolecules causing dysfunction, and subsquently, cause cell death. Peroxidative activation of these drugs could be




Scheme-1. Formation of phenoxy radicals from VP-16 and VM-26 during peroxidase activation, and a proposed pathway for the direct formation of the reactive o-quinone derivatives of VP-16 and VM-26 and $^{\cdot}CH_3$. The resulting reactive intermediates may then bind to cellular proteins and DNA.

complemenatry to that catalyzed by cytochrome P-450 and may be critical for the drug-induced cytotoxicity in tumor cells with low cytochrome P-450 activity.

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