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Cytochrome P-450-mediated *O*-Demethylation: A Route in the Metabolic Activation of Etoposide (VP-16-213)

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ABSTRACT

The antitumor agent VP-16-213 is oxidatively *O*-demethylated by rat liver microsomes and purified rat liver microsomal cytochrome P-450. 3-Methylcholanthrene can quantitatively induce *O*-demethylation of VP-16-213. The K_m and V_{max} values for *O*-demethylation by noninduced, phenobarbital-, and 3-methylcholanthrene-induced rat liver microsomes were found to be 130, 600, and 160 μM and 8.5, 11.8, and 15.6 nmol H_2CO/min·mg protein, respectively. Mass spectrometric comparison of the product of *O*-demethylation of VP-16-213 with the synthetic metabolite resulted in identification of the orthodihydroxy derivative.

In studies on the biological activity of the orthodihydroxy derivative, it was found to inactivate single- and double-stranded ΦX174 DNA, to bind to calf thymus DNA and to be highly toxic against chinese hamster ovary cells.

INTRODUCTION

The precise molecular mechanism of action of the antineoplastic agent etoposide or VP-16-213 (Fig. 1) is unknown. Loike and Horwitz (1) studied its effect on HeLa cell DNA and found that VP-16-213 caused DNA single-strand breaks. More recently, Wozniak and Ross (2) reported that the cytotoxicity of VP-16-213 in L-1210 cells is probably caused by DNA damage. A main hypothesis about the mechanism of action is that VP-16-213 interferes with the breakage-reunion reaction of mammalian topoisomerase II. Ross *et al.* (3) found that VP-16-213 stimulated DNA cleavage when incubated with purified type II topoisomerase and DNA, by induction of the stabilization of a DNA-topoisomerase II complex. Another possible mechanism is that VP-16-213 is metabolized in the dimethoxyphenol ring (the E-ring or pendant ring) to products which can cause DNA damage (1). Since it has been reported that isolated purified DNA is not broken down by the parent drug (1, 4) and that the effect of VP-16-213 on DNA requires the presence of the phenol group of the pendant ring (1), transformations of the dimethoxyphenol ring of VP-16-213 to products which inactivate DNA could also be involved in its mechanism of action, besides the inhibition by VP-16-213 itself of topoisomerase. We observed cytochrome P-450-mediated covalent binding of VP-16-213 to rat liver and HeLa cell microsomal proteins, the dimethoxyphenol ring of VP-16-213 probably being involved in the binding process (5). Support for our hypothesis of metabolic activation of VP-16-213 by transformations of the dimethoxyphenol ring is given by our recent finding that, in contrast to VP-16-213 itself, the orthoquinone derivative of VP-16-213 and its reduction product (both potential E-ring derived metabolites) cause inactivation of ΦX174 DNA (4). In a preliminary study, we observed that incubation of VP-16-213 with a reconstituted cytochrome P-450 system (oxygenation) or with cytochrome P-450 and cumene hydroperoxide (peroxygenation) resulted in *O*-demethylation of VP-16-213 (6). Sinha *et al.* (7) have recently reported that VP-16-213 was *O*-demethylated by mouse liver microsomes, but provided no mass spectrometric data on the product of *O*-demethylation. The aim of the present study was to investigate the *O*-demethylation of VP-16-213 by (*a*) control, phenobarbital-, and 3-methylcholanthrene-induced rat liver microsomes and (*b*) a reconstituted rat liver microsomal cytochrome P-450 system, and further, to identify the product of *O*-demethylation and to study the biological properties of the latter product. The results indicate that *O*-demethylation of VP-16-213 can be quantitatively induced by 3-methylcholanthrene. The product of *O*-demethylation has been identified as the orthodihydroxy derivative of VP-16-213. This orthodihydroxy derivative of VP-16-213 inactivates ΦX174 DNA, binds to calf thymus DNA, and is highly toxic to Chinese hamster ovary cells.

MATERIALS AND METHODS

Drugs and Chemicals. VP-16-213 was a gift from the Bristol Myers Company (Syracuse, NY). [^{3}H]VP-16-213 (s.a., 200 mCi/mmol) was purchased from Moravek Biochemicals, Brea, CA. The orthoquinone of VP-16-213 was synthesized by controlled potential electrolysis of VP-16-213 at a Pt-gauze electrode (8). The orthodihydroxy derivative of VP-16-213 (the catechol) was synthesized from the orthoquinone of VP-16-213 by reduction with ascorbic acid. The structure of the catechol of VP-16-213 was analyzed by mass spectrometry and nuclear magnetic resonance spectrometry (see spectral measurements). Assignments of proton resonances in the nuclear magnetic resonance spectrum of the synthetic catechol are as follows: 3.88 (s, 3, 5′-OCH_3), 4.88 (d, 1, H_4), 5.20 (s, 1, 3′-OH), 5.30 (s, 1, 4′-OH), 5.72 (s, 1, $H_{6'}$), 5.98 (s, 2, O-CH_2-O), 6.50 (s, 1, H_8), 6.68 (s, 1, $H_{2'}$), 6.78 (s, 1, H_5). The signals of 3′-OH and 4′-OH disappear upon addition of D_2O. For comparison, the assignments of proton resonances in the NMR spectrum of VP-16-213 are: 3.76 (s, 6, 3′,5′-OCH_3), 4.88 (d, 1, H_4), 5.36 (s, 1, 4′-OH), 5.96 (s, 2, O-CH_2-O), 6.22 (s, 2, $H_{2'}$ and $H_{6'}$), 6.52 (s, 1, H_8), 6.80 (s, 1, H_5). Assignments of signals in the mass spectra of the synthetic catechol are as follows; field desorption mass spectrum: *m/z* 574 (molecular ion peak, 100%), electron impact mass spectrum: *m/z* 574 (molecular ion peak, 20%), and *m/z* 368 (fragment formed by loss of the glucopyranoside moiety and OH, 100%).

^{3}H-labeled orthoquinone and catechol were synthesized by electrochemical oxidation of ^{3}H-labeled VP-16-213 (5.88 mCi/mmol) and reduction by ascorbic acid of the ^{3}H-labeled orthoquinone. The ^{3}H-labeled orthoquinone and catechol had a specific activity of 2.76 and 1.97 mCi/mmol, respectively.

Sodium phenobarbital was purchased from Brocacef B. V., Maarssen, The Netherlands. 3-Methylcholanthrene was purchased from Eastman Kodak Co., Rochester, NY. Calf thymus DNA (type I) was obtained from Sigma Chemical Company, St. Louis, MO. All other chemicals used were reagent grade.

Rat Liver Microsomes and Purified Cytochrome P-450. Liver micro-

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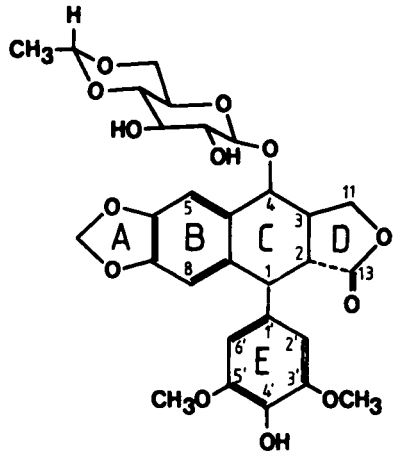

Fig. 1. Structure of VP-16-213.

somes were prepared from male albino Wistar rats (180–200 g) by the previously described method (5). Microsomes from Wistar rats pretreated with phenobarbital (1 g/liter drinking water for 10 days) or 3-methylcholanthrene (40 mg/kg/2 ml arachidis oil by i.p. injection 1 × daily for 3 days) were prepared in the same way. Cytochrome P-450 and cytochrome P-450 reductase were purified from phenobarbital-induced rat liver microsomes by the method described by Waxman *et al.* (9). The specific activity of the purified cytochrome P-450 and cytochrome P-450 reductase was 14 nmol/mg protein and 24 units/mg protein, respectively.

Incubation of VP-16-213 with purified cytochrome P-450 (oxygenation) consisted of 47 nmol VP-16-213, 0.24 nmol cytochrome P-450b, 0.1 unit cytochrome P-450 reductase, 6 μg 1-α-dilauroylphosphatidylcholine and 5×10^{-2} M Tris-HCl (pH 7.4), in a total volume of 350 μl. The reaction was started by addition of 350 nmol NADPH. The mixture was incubated at 37°C for 10 min, protein precipitated by addition of 175 μl 20% TCA[2] and the amount of formaldehyde formed by *O*-demethylation determined by the method of Nash (10).

Incubation of VP-16-213 with purified cytochrome P-450 (peroxygenation) consisted of 45 nmol VP-16-213, 0.44 nmol cytochrome P-450b, 17.5 μg 1-α-dilauroylphosphatidylcholine, 350 nmol of the oxygen donor cumene hydroperoxide and 5×10^{-2} M Tris-HCl (pH 7.4), in a total volume of 350 μl. The reaction was started by addition of cumene hydroperoxide, followed by incubation for 10 min at 37°C, protein precipitation by the addition of 175 μl 20% TCA and determination of the amount of formaldehyde formed.

Incubation of VP-16-213 with noninduced, phenobarbital-induced, and 3-methylcholanthrene-induced rat liver microsomes consisted of 0–170 nmol VP-16-213, 2.4 mg microsomal protein, 100 μmol Tris-HCl (pH 7.4), 8.4 μmol $MgCl_2 \cdot 6H_2O$, 8.4 μmol glucose-6-phosphate, 2.0 μmol semicarbazide, and 0.67 unit of glucose-6-phosphate dehydrogenase, in a total volume of 2 ml. The reaction was started by the addition of 1 μmol NADP. The mixture was incubated for 10 min at 37°C, protein precipitated by addition of 1 ml 20% TCA and the amount of formaldehyde formed determined as described above. The catechol of VP-16-213 was isolated from the incubation mixture after protein precipitation as described under high performance liquid chromatography.

High Performance Liquid Chromatography. High performance liquid chromatography was performed using a Waters 6000A solvent delivery system, in combination with an ESA Model 5100A coulochem electrochemical detector and a CP-Microspher 3-μm C18 column (100 × 4.6 mm) with a 10- × 2.1-mm precolumn packed with Baker 40 μm C18. The mobile phase consisted of methanol/water (40/60), 0.1 M citrate buffer (pH 7.0). Electrochemical detection of orthoquinone of VP-16-213 and catechol of VP-16-213 or VP-16-213 itself were performed at a potential of −250 mV and +200 mV, respectively.

Isolation of the catechol of VP-16-213 was performed by preconcentration of a 3 × 0.5-ml aqueous incubation mixture after protein precipitation. The precolumn was eluted with a mixture of distilled, spectroscopically pure methanol and water (40/60). At the retention time of the synthetic catechol the electrochemical detector was switched off, the eluent containing the peak collected and extracted with 10 ml distilled spectroscopically pure chloroform, the chloroform layer removed and evaporated to dryness under N_2. The residue was subjected to mass spectrometry.

Spectral Measurements. Proton nuclear magnetic resonance measurements were performed on a 90-MHz Bruker WH-90 spectrometer using tetramethylsilane as an internal reference. Samples were dissolved in $CDCl_3$.

Field desorption mass spectrometric measurements were performed on a double-focusing Varian MAT 731 mass spectrometer equipped with an EI/field isonization/field desorption source and interfaced to a SS300 data system. The emitter temperature was programmed with an emission control unit. High resolution (10^4) electron impact measurements were performed by peak matching and computer controlled high voltage scanning on a Finnigan MAT 8230 mass spectrometer, using a direct probe inlet system with perfluorokerosene as the reference substance.

Cytotoxicity Experiments. Chinese hamster ovary cells (Aux-B1) were grown in monolayer culture at 37°C in α-minimal essential medium containing 10% fetal calf serum. Rat hepatoma cell line (Reuber H35) was maintained in Dulbecco's minimum essential medium containing 10% fetal calf serum. Cells at concentrations of 1–5.10^4/ml were added to 2-ml wells on day one. On the second day the medium in each well was replaced by medium containing various concentrations of drug (VP-16-213 or the catechol of VP-16-213). The incubation at 37°C was continued for 3 days. Surviving cells were suspended with trypsin-EDTA and counted with a cell-counter/hemocytometer.

Average counts for drug-treated cells were expressed as a percentage of the average number of counts for untreated control cells. The IC_{50} value is the concentration of drug giving a 50% value.

The cytochrome P-450 content of the microsomal fraction of the cells was determined by difference spectrophotometry (11).

Incubations with ss and RF ΦX174 DNA. Details concerning the preparation of single- and double-stranded DNA and determination of its biological activity (spheroplast test) have essentially been described by Blok *et al.* (12).

Solutions of single- or double-stranded ΦX174 DNA (1.7×10^{-1} μg/ml) and 5×10^{-2} M potassium phosphate, pH 7.4, were incubated at 37°C in the presence of the catechol of VP-16-213 or VP-16-213 itself (2.2×10^{-4} M). At several time intervals of incubation, samples were taken and diluted 20-fold with ice-cold Tris buffer (0.025 M, pH 8.2) to stop the reaction. Diluted reaction samples (0.1 ml) were mixed with an equal volume of spheroplasts of *Escherichia coli* K12 (AB 1157). After 10 min at 20°C, 0.8 ml of LBM [Luria Broth also containing 10% (w/v) sucrose, 0.1% (w/v) glucose, and 0.2% (w/v) $MgCl_2$] medium was added and incubation was continued (37°C) for 2 h. After this 4 ml of cold distilled water was added and the bacteriophage titers were determined by plating using *E. coli* C as the indicator bacterium.

Binding of ^{3}H-labeled VP-16-213 and Catechol to Calf Thymus DNA. ^{3}H-Labeled compound (170×10^{-6} M, 2100 dpm/μg DNA) and DNA (100 μg/ml) were incubated for 1 h at 37°C in 5×10^{-2} M potassium phosphate, pH 7.4, in a volume of 2 ml. The DNA-drug complexes were isolated using a modification of the procedure of Leadon and Cerutti (13). After incubation, the DNA-drug complex was precipitated by addition of 200 μl 3 M sodium acetate and 6 ml ethanol (stored at −20°C). The precipitate was stored at −70°C for 30 min and isolated by filtration over glass fiber filters (i.d., 25 mm; pore size, 1.0 μm). The incubation valve was washed with 1 ml ethanol and the filter washed three times with 2.5 ml ethanol. The filter was irradiated with 15 Gy, using a ^{60}Co γ-ray source (Gamma cell 100; Atomic Energy of Canada Ltd.), after which an aqueous wash with 5 ml H_2O released the DNA. The DNA-drug complexes in the samples were measured with a Betamatic scintillation counter (Kontron Analytical International, Zürich, Switzerland) using Opti-Fluor (United Technologies Packard Instrument Company, IL) as the counting scintillant.

[2] The abbreviations used are: TCA, trichloroacetic acid; HPLC, high performance liquid chromatography; EI, electron impact; ss, single-stranded; RF, replicative form.

RESULTS

***O*-Demethylation of VP-16-213 to the Orthodihydroxy Derivative.** The chemical shift assignments from the 90-MHz proton nuclear magnetic resonance spectrum of VP-16-213 are in agreement with those reported by Jardine *et al.* (14). The intensity of the methoxy peak in the spectrum of the synthetic catechol was half of that of the corresponding peak in the spectrum of VP-16-213. For the protons at the 2′ and 6′ positions, which gave rise to one band in the spectrum of VP-16-213, two separate peaks were found in the spectrum of the catechol. The spectrum of VP-16-213 showed one resonance signal of the 4′-OH group. The spectrum of the catechol showed two resonance signals of the 3′- and 4′-OH groups, which disappeared upon addition of D_2O. The other protons of VP-16-213 and its catechol showed the same chemical-shift assignments and intensities.

In the high resolution electron impact mass spectrum of the synthetic catechol the molecular ion peak at *m/z* 574 was detected with an intensity of approximately 20%. The base peak in the EI mass spectrum was the ion at *m/z* 368. That fragment is the product of the loss of the glucopyranoside moiety and OH. The EI mass spectrum of VP-16-213 had a base peak at *m/z* 382. This peak corresponds to the same fragment as in the EI spectrum of the catechol, but is 14 mass units higher due to the presence of an OCH_3-group instead of the OH-group in VP-16-213. The field desorption mass spectrum of the synthetic catechol showed the molecular ion peak at *m/z* 574 as the base peak.

The above nuclear magnetic resonance and mass spectrometric data show that the synthetic catechol possesses the structure proposed in Fig. 2.

Incubation of VP-16-213 with cytochrome P-450b (inducible by phenobarbital), cytochrome P-450 reductase and NADPH (reconstituted system), as well as incubation with cytochrome P-450b and cumene hydroperoxide (peroxygenation) resulted in *O*-demethylation of VP-16-213. The rates of formaldehyde production by these systems were found to be 1.9 and 0.8 nmol H_2CO/min · nmol P-450b, respectively. *O*-demethylation of VP-16-213 by rat liver microsomes was also studied, using noninduced, phenobarbital-, and 3-methylcholanthrene-induced microsomes. Fig. 3 shows the Lineweaver-Burk plots for the *O*-demethylation of VP-16-213 by these systems. The K_m and V_{max} values calculated from the Lineweaver-Burk plots are shown in the legends to Fig. 3.

After incubations with both purified cytochrome P-450 and microsomes, HPLC-analysis revealed the presence of an electrochemically oxidizable product with a retention time identical to that of the synthetic catechol of VP-16-213 (Fig. 4, *A* and *B*). The product could not be electrochemically reduced. The corresponding product obtained from an incubation of VP-16-213 with phenobarbital-induced rat liver microsomes, was subjected to mass spectrometry. The high resolution EI mass spectrum of the new metabolite shows the presence of two main ions: *m/z* 368.091 (theoretical, 368.0896 = $C_{20}H_{16}O_7$; Fig. 5*A*)

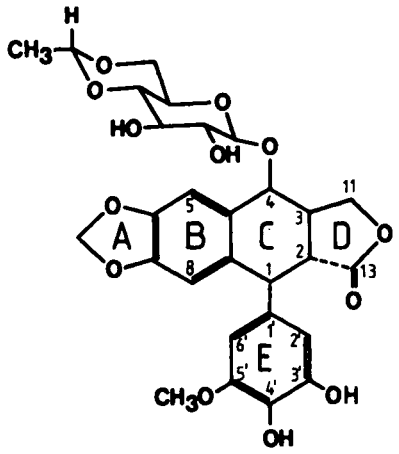

Fig. 2. Structure of the catechol of VP-16-213.

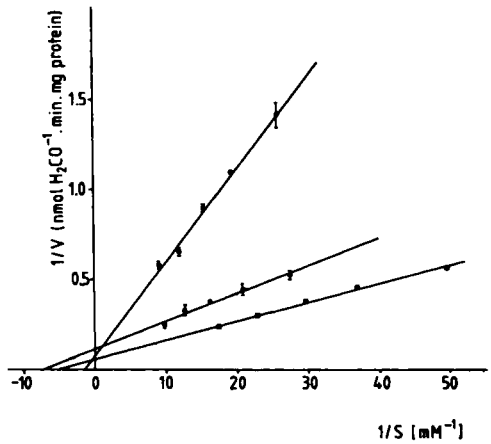

Fig. 3. Lineweaver-Burk plots for *O*-demethylation of VP-16-213 by noninduced (●), phenobarbital-induced (○), and 3-methylcholanthrene-induced (■) rat liver microsomes. *Values*, mean of three determinations. K_m and V_{max} values calculated from the Lineweaver-Burk plots for control, phenobarbital-induced, and 3-methylcholanthrene-induced microsomes are: K_m = 130, 600, and 160 μM; V_{max} = 8.5, 11.8, and 15.6 nmol H_2CO/min · mg protein, respectively.

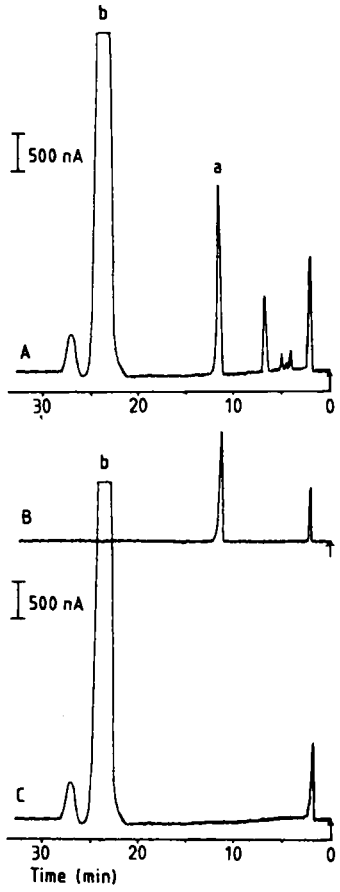

Fig. 4. *A*, chromatogram resulting from HPLC analysis of a mixture, obtained by incubation of VP-16-213 with purified cytochrome P-450. Metabolite (*a*); VP-16-213 (*b*). *B*, chromatogram of the synthetic catechol of VP-16-213. *C*, control chromatogram of incubation of VP-16-213 without cytochrome P-450.

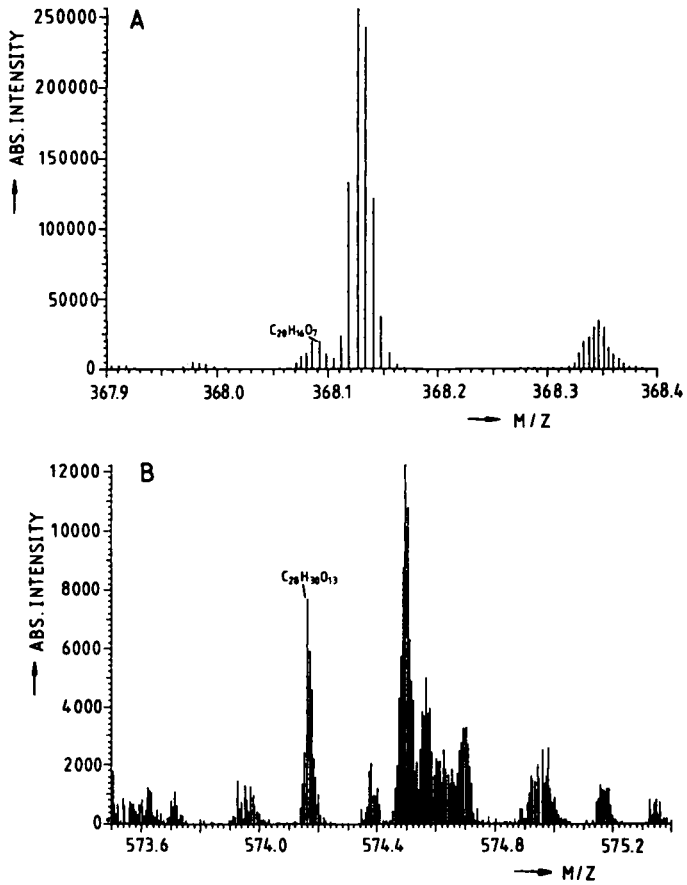

Fig. 5. High resolution electron impact mass spectra (without background substraction) of the metabolite, showing the major fragment at *m/z* 368 formed by loss of the glucopyranoside moiety and OH (*A*), and the molecular ion at *m/z* 574 (*B*).

and *m/z* 574.1681 (theoretical, 574.1685 = $C_{28}H_{30}O_{13}$; Fig. 5*B*). In the EI mass spectrum of the synthetic catechol, the same signals are present (see above). The mass spectrometric data on the metabolite, combined with its electrochemical behavior and the fact that the metabolite has the same retention time as the synthetic catechol, prove that the metabolite is the catechol of VP-16-213.

Comparison of the chromatogram of incubation of VP-16-213 and cytochrome P-450 with the control chromatogram of incubation of VP-16-213 without cytochrome P-450 shows that only the peak with longer retention time than VP-16-213 is also present besides VP-16-213 in the control chromatogram (Fig. 4*C*); this component could be a decomposition product of VP-16-213.

Studies on the Biological Activity of the Orthodihydroxy Derivative. The cytotoxicity experiments with VP-16-213 and the synthetic catechol revealed that the IC_{50} values for Aux-B1 cells were 0.7 and 0.1 μM, respectively, and for H35 cells 5 and 30 μM, respectively. The cytochrome P-450 content determined in 10^7 cells was low: 26 pmol/mg protein and 120 pmol/mg protein for Aux-B1 and H35 cells, respectively. For comparison, the cytochrome P-450 content of rat liver microsomes is 920 pmol/mg protein. The IC_{50} data show that in Aux-B1 cells, the catechol of VP-16-213 has a higher cytotoxicity than VP-16-213 itself.

The results of the experiments on the binding of ^{3}H-labeled VP-16-213 and catechol to calf thymus DNA are as follows. The binding ratios for VP-16-213 and the catechol were 3.4 and 32.9 dpm/μg DNA, respectively. Thus, the catechol showed about 10 times more binding to calf thymus DNA than the parent molecule VP-16-213.

As test system for inactivation of DNA by the catechol of VP-16-213, biologically active ΦX174 DNA was used. Fig. 6 shows the survival curves for inactivation of ss (single-stranded) and RF (double-stranded) ΦX174 DNA. With both DNAs clear inactivation was found in the presence of catechol. The experimental data are fitted to an exponential survival. However, a slightly bending curve cannot be excluded. Incubation of ss and RF ΦX174 DNA with VP-16-213 did not result in a decrease in DNA activity, indicating the absence of biologically important DNA damage and the necessity of activation of VP-16-213 to cause DNA damage.

DISCUSSION

O-Demethylation of VP-16-213 by mouse liver microsomes has recently been reported by Sinha *et al.* (7). These authors showed the production of formaldehyde by *O*-demethylation, but did not identify the product formed from VP-16-213. The present studies are the first to show that the product of *O*-demethylation of VP-16-213 is its orthodihydroxy derivative or catechol. Both the reconstituted cytochrome P-450 system and the peroxygenative system consisting of cytochrome P-450 and the oxygen donor cumene hydroperoxide were capable of *O*-demethylation of VP-16-213. Production of formaldehyde by the reconstituted system was about 2-fold higher than by the peroxygenative system. The V_{max} for *O*-demethylation of VP-16-213 by rat liver microsomes increased in the following order: noninduced < phenobarbital-induced < 3-methylcholanthrene-induced rat liver microsomes. The K_m-values for *O*-demethylation by noninduced and 3-methylcholanthrene-induced microsomes are comparable, whereas the K_m value for *O*-demethylation by phenobarbital-induced microsomes is about four times higher. These data indicate that 3-methylcholanthrene can quantitatively induce *O*-demethylation of VP-16-213, and that the affinity of VP-16-213 for the cytochrome P-450 in noninduced and 3-methylcholanthrene-induced microsomes is higher than for the enzyme in phenobarbital-induced microsomes.

The product of *O*-demethylation of VP-16-213 by rat liver microsomes was identified as its orthodihydroxy derivative by

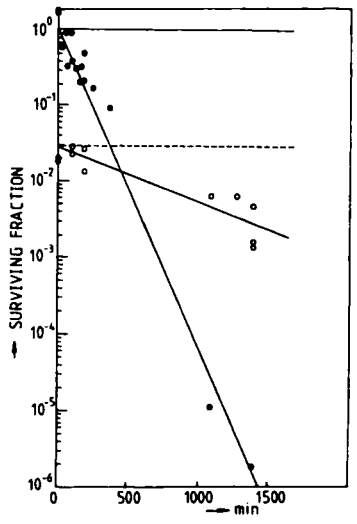

Fig. 6. Survival curves for single-stranded (●, ——) and double-stranded (○, - - -) ΦX174 DNA (both 1.7×10^{-1} μg/ml) dissolved in 5×10^{-2} M phosphate buffer (pH 7.4), in the presence of 2.2×10^{-4} M catechol of VP-16-213 (●, ○) or the parent compound VP-16-213 (——, - - -).

the combination of mass spectrometric analysis, similarity in retention time with the synthetic catechol and electrochemical behavior. The formation of the orthodihydroxy derivative by purified cytochrome P-450 was indicated by similarity in retention time with the synthetic catechol and electrochemical behaviour.

Studies on the biological properties of the catechol of VP-16-213 showed that it could well be an important metabolite of VP-16-213. The catechol of VP-16-213 showed cytotoxicity against Aux-B1 and H35 cells. The catechol of VP-16-213 showed 10 times more extensive binding to calf thymus DNA than VP-16-213 and, most importantly, the catechol showed in contrast to VP-16-213 itself inactivation of both single-stranded and double-stranded ΦX174 DNA. These findings give further support for the hypothesis that the cytotoxicity of VP-16-213 is based upon metabolic activation of the E-ring of VP-16-213 to products which can cause DNA damage. This hypothesis is also supported by the recent finding of Teicher *et al.* (15) that VP-16-213 cytotoxicity *in vitro* and *in vivo* is greatly enhanced in oxygenated compared with hypoxic tissue. This is in line with oxygen-dependent oxidation of VP-16-213 by cytochrome P-450 to the catechol metabolite. The authors suggested that VP-16-213 acts through an oxidation-reduction process involving the production of oxygen free radicals. Our results suggest that the metabolite involved in the oxidation-reduction process (the catechol) might itself be responsible for DNA damage. The original finding of Loike and Horwitz that the presence of the 4′—OH group is necessary for DNA-inactivation was recently confirmed by Long *et al.* (16) who studied the DNA breakage activity of several congeners of podophyllotoxin. This gives further support for the importance of catechol formation from VP-16-213 for DNA-inactivation by the drug. Sinha (7) reported that VP-16-213, but not podophyllotoxin, inhibited daunomycin-promoted microsomal lipid peroxidation, suggesting that the presence of the 4′—OH group of VP-16-213 is also necessary for this antioxidant effect of VP-16-213. The catechol of VP-16-213 could play a role in this process by chelating iron or other metal ions that may be required for lipid peroxidation (17).

Further evaluation of the biological properties of the catechol of VP-16-213, in particular *in vivo* investigation of its antitumor properties, is needed.

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