

EFFECTS OF THE ORTHO-QUINONE AND CATECHOL OF THE ANTITUMOR DRUG VP-16-213 ON THE BIOLOGICAL ACTIVITY OF SINGLE-STRANDED AND DOUBLE-STRANDED Φ X174 DNA

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Abstract—We have studied the effects of the recently reported two new metabolites of the antitumor agent VP-16-213, the ortho-dihydroxy derivative or catechol and the ortho-quinone, on the biological activity of single-stranded and double-stranded Φ X174 DNA, the binding of the metabolites to calf thymus DNA and the conversion of the catechol into the ortho-quinone. Evidence was obtained for the oxidation of the catechol into the ortho-quinone and for the fact that the ortho-quinone is the metabolite of VP-16-213 responsible for its binding to rat liver microsomal proteins. The catechol and ortho-quinone of VP-16-213 were found to bind 7–9 times more strongly to calf thymus DNA than VP-16-213 itself. In contrast to the parent compound VP-16-213, the catechol as well as the ortho-quinone inactivated both single-stranded (ss) and double-stranded (RF) biologically active Φ X174 DNA. The mean T_{37} -values for inactivation of ss and RF Φ X174 DNA by 2.2×10^{-4} M catechol at 37° and pH 7.4 were 96 and 640 min, respectively. Reduction of the ortho-quinone by NADPH cytochrome P-450 reductase resulted in formation of the catechol. The system ortho-quinone/NADPH cytochrome P-450 reductase inactivated ss Φ X174 DNA with a mean T_{37} -value of 454 min, and this inactivation was inhibited by DMSO. The mean T_{37} -value for inactivation of ss Φ X174 DNA by 1.8×10^{-4} M ortho-quinone at 37° and pH 4.0 was 24 min. The chemical stability of the ortho-quinone and the extent of inactivation of ss Φ X174 DNA by the ortho-quinone were both pH-dependent: at higher pH the ortho-quinone was less stable and gave less inactivation of DNA. The aqueous decomposition product(s) of the ortho-quinone formed at pH 7.4 inactivated ss Φ X174 DNA with a mean T_{37} -value of 175 min. The rate of inactivation of RF Φ X174 DNA by the ortho-quinone at pH 4.0 was twice as low as the rate of inactivation of ss Φ X174 DNA: $T_{37} = 49$ min. When using excision repair deficient *E. coli* mutants (*uvrA*[−] or *uvrC*[−]), a higher inactivation of RF Φ X174 DNA was found: $T_{37} = 29$ min for *uvrA*[−] *E. coli*, indicating that a part of the DNA damage introduced by the incubation with ortho-quinone is removed by excision repair. Neutral and alkaline sucrose gradient centrifugation of ss Φ X174 DNA incubated with the ortho-quinone at pH 4.0 revealed the absence of DNA-breakage, suggesting that introduction of strand breaks and alkali-labile sites does not play a role in the inactivation of DNA by the ortho-quinone. Adduct-formation probably plays a role in the inactivation of DNA by this metabolite.

VP-16-213 [4'-demethylepipodophyllotoxin-1-(4,6-*O*-ethylidene- β -D-glucopyranoside), NSC 141540, etoposide, Fig. 1] is an important antineoplastic agent used against several types of tumors [1]. The cytotoxicity of VP-16-213 is probably caused by the introduction of DNA damage. The main types of DNA damage identified so far are DNA single strand breaks, DNA double strand breaks and DNA-protein cross-links [2]. Many indications were obtained that the introduction of at least a part of these DNA-lesions can be explained by the capacity of this antitumor drug to interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II by stabilizing a cleavable complex [3]. Therefore, it

has been proposed that the principal mechanism of cytotoxic action of etoposide is the "poisoning" of DNA topoisomerase II, probably by its ability to bind reversibly to this enzyme. On the other hand, indications that besides interaction with DNA topoisomerase, VP-16-213 may have other mechanisms of action came from biotransformation studies, which show that oxidation of the dimethoxyphenol ring (the E-ring, Fig. 1) can take place leading to products which are able to cause DNA-damage [4, 5]. That metabolic activation may be a requirement for the cytotoxic and DNA damaging effect of VP-16-213 was first suggested by Loike and Horwitz, who showed that etoposide caused intracellular degradation of HeLa cell DNA, but that incubation of purified DNA with the drug did not lead to detectable DNA damage [6]. In addition, they showed that the presence of the 4'-OH group in the E-ring was necessary for the intracellular DNA-damaging effect

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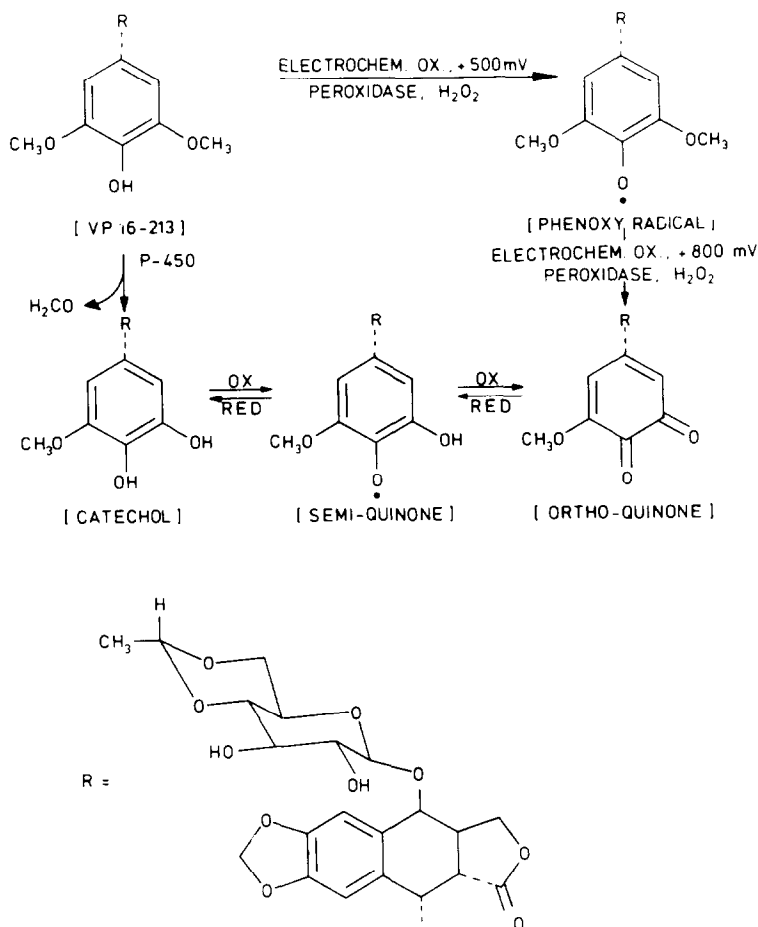


Fig. 1. Possible conversions of the dimethoxyphenol ring of VP-16-213 leading to the catechol and ortho-quinone of VP-16-213.

of the drug. Wozniak and Ross observed that dehydrogenase inhibitors like disulfiram and diethyldithiocarbamate, free radical scavengers such as sodium benzoate and dehydrogenase substrates prevent the VP-16-213 induced production of strand breaks and cytotoxicity in L1210 cells, suggesting a role of a dehydrogenase in an oxidation-reduction type of reaction [7]. That oxidation-reduction processes are involved in the cytotoxic action of VP-16-213 was recently confirmed by Teicher *et al.*, who reported that VP-16-213 cytotoxicity is greatly enhanced towards oxygenated EMT6 cells as compared towards hypoxic cells [8]. We, as well as Sinha [9] and Haim *et al.* [10], recently reported that VP-16-213 undergoes *O*-demethylation by rat and mouse liver microsomes and purified rat liver cytochrome P-450 and identified the product of *O*-demethylation as the ortho-dihydroxy derivative or catechol of VP-16-213 [5, 9–11]. In preliminary studies, the catechol and the ortho-quinone of VP-16-213—also a possible product of peroxidative metabolism of VP-16-213 [12]—were found to inactivate Φ X174 DNA, in con-

trast to VP-16-213 itself [4, 5]. These observations are in favour of the hypothesis that oxidative biotransformations of the E-ring of VP-16-213 may play an important role in DNA inactivation.

The aims of the present investigation were (a) to study the conversion of the catechol of VP-16-213 into the ortho-quinone and the stability of both metabolites, (b) to study the binding of ortho-quinone and catechol to microsomal proteins and calf thymus DNA, and (c) to investigate in detail the inactivation of Φ X174 DNA by the ortho-quinone and the catechol of VP-16-213. The results show that the catechol of VP-16-213 can be oxidized into the ortho-quinone; both ortho-quinone and catechol bind strongly to calf thymus DNA; ortho-quinone as well as catechol inactivate ss|| and RF Φ X174 DNA; the inactivation of Φ X174 DNA by the ortho-quinone is probably based on adduct formation. The conversions of the dimethoxyphenol ring of VP-16-213 leading to the catechol and ortho-quinone are shown in Fig. 1.

MATERIALS AND METHODS

Drugs and chemicals

VP-16-213 was a gift from the Bristol Myers Com-

|| Abbreviations used: ss, Single-stranded; RF, replicative form; HPLC, high performance liquid chromatography; DPE, 1,2-diphenylethylenediamine.

pany (Syracuse, NY). ^3H -VP-16-213 (sp. act. 200 mCi/mmol) was purchased from Moravak Biochemicals (Brea, CA). The ortho-quinone of VP-16-213 was synthesized by controlled potential electrolysis of VP-16-213 at a Pt-gauze electrode [13]. The ortho-dihydroxy derivative of VP-16-213 (the catechol) was synthesized from the ortho-quinone of VP-16-213 by reduction with ascorbic acid [5]. ^3H -labelled ortho-quinone (sp. act. 4.78 mCi/mmol) was synthesized by electrochemical oxidation of ^3H -labelled VP-16-213 diluted with cold VP-16-213 to 5.88 mCi/mmol and ^3H -labelled catechol (3.97 mCi/mmol) by reduction of ^3H -labelled ortho-quinone with ascorbic acid.

1,2-Diphenylethylenediamine (DPE) was synthesized as described by Irving *et al.* [14]. The quinaxaline of the ortho-quinone of VP-16-213 was synthesized by reaction of an excess DPE with the ortho-quinone of VP-16-213 ($8 \times 10^{-4}\text{M}$) in chloroform. The quinaxaline of the ortho-quinone of VP-16-213 was isolated by preparative thin layer chromatography on reversed-phase silica plates and subjected to field desorption mass spectrometry (see spectral measurements). The mass spectrum showed a MH^+ peak at m/z 749 (30%), characteristic for the quinaxaline of the ortho-quinone of VP-16-213. Ethylenediamine was purchased from J. T. Baker Chemicals B.V. (Deventer, The Netherlands). Potassium superoxide and calf thymus DNA (type I) were purchased from Brocacef B.V. (Maarssen, The Netherlands). All other chemicals used were reagent grade.

Rat liver microsomes and purified cytochrome P-450 reductase

Liver microsomes were prepared from male albino Wistar rats (180–200 g) by the previously described method [15]. Microsomes from Wistar rats pretreated with phenobarbital (1 g/l in drinking water for 10 days) were prepared in the same way. Cytochrome P-450 reductase was purified from phenobarbital-induced rat liver microsomes by the method described by Waxman *et al.* [16].

Covalent microsomal protein binding experiments of ^3H -labelled ortho-quinone or ^3H -labelled VP-16-213 were carried out as described previously [15]. The binding of ^3H -VP-16-213 or ^3H -ortho-quinone ($1.7 \times 10^{-5}\text{M}$, 4.9×10^5 dpm/incubation) to microsomal proteins was studied in presence or absence of $1 \times 10^{-3}\text{M}$ NADPH. The degree of binding of ^3H -VP-16-213 to microsomal proteins was also investigated in the presence of $1 \times 10^{-2}\text{M}$ ethylenediamine or $1 \times 10^{-3}\text{M}$ ascorbic acid, and the degree of binding of ^3H -ortho-quinone also in the presence of $1 \times 10^{-2}\text{M}$ DPE.

To investigate the formation of the quinaxaline of the ortho-quinone of VP-16-213 in incubations of VP-16-213 with rat liver microsomes, $1.7 \times 10^{-4}\text{M}$ VP-16-213 was incubated in a volume of 20 ml with microsomal protein (20 mg), Tris-HCl pH 7.4 ($5 \times 10^{-2}\text{M}$), magnesium chloride ($4.2 \times 10^{-3}\text{M}$), glucose-6-phosphate ($4.2 \times 10^{-3}\text{M}$), glucose-6-phosphate-dehydrogenase (8.9 I.U.) and NADP ($5 \times 10^{-4}\text{M}$). After 10 min incubation at 37° , $2.8 \times 10^{-3}\text{M}$ DPE was added and the incubation continued for 15 min, followed by the addition of

20 ml cold methanol (stored at -20°). After centrifugation, the methanol was evaporated and the residue extracted with 20 ml spectroscopically pure chloroform. The chloroform was evaporated and the residue subjected to HPLC.

High performance liquid chromatography

HPLC was performed using a Waters 6000A Solvent Delivery System in combination with an ESA Model 5100A Coulchem electrochemical detector, a Perkin-Elmer LC-75 Spectrophotometric Detector and a CP-Microspher $3\mu\text{m}$ C18 column ($100 \times 4.6\text{mm}$). The mobile phase consisted of methanol/water (40/60 or 50/50 v/v), the flow rate was 1 ml/min. UV detection at a wavelength of 254 nm was employed. Electrochemical detection of ortho-quinone of VP-16-213 and catechol of VP-16-213 or VP-16-213 itself were performed at a potential of -250mV and $+200\text{mV}$, respectively.

Spectral measurements

Field desorption mass spectrometric measurements were performed on a double focusing Varian MAT 731 mass spectrometer equipped with an EI/FI/FD source and interfaced to a SS300 data system. The emitter temperature was programmed with an emission control unit. Spectrophotometric measurements were performed with a Beckman model 35 spectrophotometer.

Binding of ^3H -labelled ortho-quinone and catechol of VP-16-213 to calf thymus DNA

^3H -labelled ortho-quinone, catechol or VP-16-213 ($1.7 \times 10^{-4}\text{M}$, 2100 dpm/ μg DNA) and DNA (100 $\mu\text{g/ml}$) were incubated for 1 hr at 37° in $5 \times 10^{-2}\text{M}$ potassium phosphate pH 4.0 or 7.4 in a volume of 2 ml. The DNA-drug complexes were isolated using a modification of the procedure of Leadon and Cerutti [17]. After incubation, the DNA-drug complex was precipitated by addition of 200 μl 3M sodium acetate and 6 ml ethanol (stored at -20°). The precipitate was stored at -70° for 30 min and isolated by filtration over glass fibre filters (i.d. 25 mm, pore size $1.0\mu\text{m}$). The incubation valve was washed with 1 ml ethanol and the filter washed 3 times with 2.5 ml ethanol. The DNA-drug complexes retained on the filter were measured with a Betamatic scintillation counter (Kontron Analytical International, Zürich, Switzerland) using Opti-Fluor as counting scintillant. Control incubations were performed in which the filter was irradiated with 15 Gy, using a ^{60}Co Γ -ray source (Gamma cell 100 Atomic Energy of Canada Ltd.) and the DNA released from the filter by washing with 5 ml H_2O , showing a recovery of the DNA of more than 95%. The binding of ^3H -labelled ortho-quinone and catechol to denatured calf thymus DNA was studied by denaturing DNA by heating at 100° for 5 min, followed by incubation with the drugs using the same conditions as described above for binding to double-stranded DNA.

Incubations with ss and RF ΦX174 DNA

(a) *Spheroplast-tests.* Single-stranded ΦX174 DNA and ΦX174 double-stranded (RF) DNA were isolated from wild-type ΦX174 bacteriophage and

Φ X174-infected *E. coli* host bacteria according to Blok *et al.* [18] and Baas *et al.* [19], respectively. Solutions of ss or RF Φ X174 DNA ($1.7 \times 10^{-1} \mu\text{g}/\text{ml}$) were incubated at 37° for increasing periods of time with (a) $5 \times 10^{-2} \text{ M}$ potassium phosphate of different pHs (pH 4, 5 or 7.4) and increasing concentration of the ortho-quinone of VP-16-213 (0.2 – $1.8 \times 10^{-4} \text{ M}$), (b) $5 \times 10^{-2} \text{ M}$ potassium phosphate pH 7.4, the ortho-quinone of VP-16-213 ($1.8 \times 10^{-4} \text{ M}$), NADPH ($1 \times 10^{-3} \text{ M}$) and cytochrome P-450 reductase (0.31 – 1.56 units) in the absence or presence of 0.5% DMSO, (c) $5 \times 10^{-2} \text{ M}$ potassium phosphate pH 7.4 and the catechol of VP-16-213 ($2.2 \times 10^{-4} \text{ M}$). At several time intervals of incubation, samples of $25 \mu\text{l}$ were taken and diluted 20-fold with ice-cold 0.025 M Tris-HCl buffer (pH 8.2) to stop the reaction. 0.1 ml diluted reaction samples were mixed with an equal volume of spheroplasts of *Escherichia coli* AB1157 (wild type) or the excision repair deficient strains AB1886 (*uvrA*⁻) or AB1884 (*uvrC*⁻) [20]. After 10 min at 20° , 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 the incubation continued at 37° for 2 hr. After this 4 ml of cold distilled water was added and the bacteriophage titers were determined by plating using *E. coli* C as indicator bacterium [21]. All results presented are corrected for effects due to pH and temperature alone; this effect is only substantial in the case of incubation of single-stranded DNA at pH 4 due to formation of apurinic sites [22].

(b) *Sedimentation in sucrose gradients.* Prior to sucrose gradient centrifugation solutions of ss Φ X174 DNA ($4 \mu\text{g}/\text{ml}$) were incubated at 37° in $5 \times 10^{-2} \text{ M}$ potassium phosphate pH 4 with the ortho-quinone of VP-16-213 ($1.4 \times 10^{-3} \text{ M}$). At several time intervals of incubation, 350 – $450 \mu\text{l}$ samples were taken. In the case of alkaline sucrose gradient centrifugation, the samples were made 0.1 M in NaOH and incubated for 30 min at 37° (final pH about 12.5) prior to sedimentation.

Linear sucrose gradients (5–40% w.v. in $1 \times 10^{-2} \text{ M}$ NaCl + $1 \times 10^{-4} \text{ M}$ EDTA + $1 \times 10^{-3} \text{ M}$ potassium phosphate buffer pH 7.2) were prepared in cellulose nitrate tubes. In the case of alkaline sucrose gradients, 5–40% w.v. sucrose in $1 \times 10^{-4} \text{ M}$ EDTA and $1 \times 10^{-2} \text{ M}$ NaOH, pH 11.6, were used. 350 – $450 \mu\text{l}$ of the incubation mixture containing 1.4 – $1.8 \mu\text{g}$ of DNA was layered on the top of the gradients. Centrifugation was performed at 15° and $36,000 \text{ r.p.m.}$ for about 19 hr in a Beckman S.W.41 rotor. After centrifugation the gradients were analyzed as described by Lafleur *et al.* [23]. From the sedimentation pattern the amount of unbroken molecules can be derived. From the exponential decrease of this amount the number of breaks can be calculated [23].

RESULTS

(a) Oxidation of the catechol of VP-16-213 into the ortho-quinone

In our previous studies we were able to show that VP-16-213 is *O*-demethylated in the E-ring by rat liver microsomes and purified rat liver cytochrome

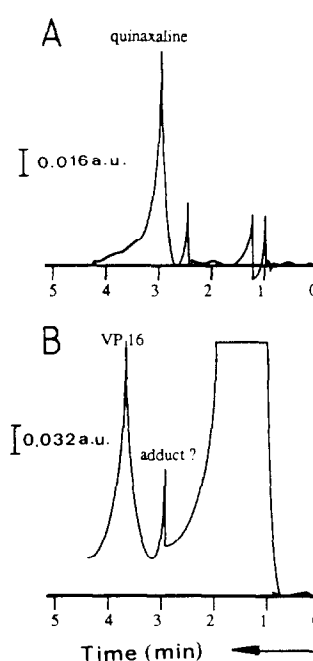


Fig. 2. HPLC chromatogram of (A) the synthetic quinaxaline of the ortho-quinone of VP-16-213 and (B) the chloroform extract of the incubation mixture of VP-16-213 with rat liver microsomes and DPE. The HPLC eluent was methanol/water (50/50 v/v).

P-450 [5, 11]. The product of *O*-demethylation could be identified as the ortho-dihydroxy derivative or the catechol. Cytochrome P-450-mediated covalent binding of VP-16-213 to microsomal proteins was previously observed in this series of studies [15]. It was suggested that the ortho-quinone, which can be formed by oxidation of the catechol, was the protein-binding species. Since it is very difficult to detect free ortho-quinone in microsomal incubations with VP-16-213 due to its high reactivity, we decided to add the ortho-quinone trapping agent 1,2-diphenylethylenediamine (DPE). This compound has been successfully applied by Nohta for the determination of catecholamines by oxidation to the ortho-quinones and trapping of the ortho-quinones with DPE to quinaxalines [24]. HPLC-analysis of the incubation mixture revealed the formation of a component with the same retention time as the synthetic quinaxaline of the ortho-quinone of VP-16-213 (Fig. 2).

To investigate whether the ortho-quinone of VP-16-213 could be the species responsible for the covalent binding of VP-16-213 to rat liver microsomal proteins, the covalent binding of VP-16-213 and ortho-quinone to microsomal proteins was studied (Table 1). The ortho-quinone of VP-16-213 was found to bind extensively to microsomal proteins in the absence of NADPH (about 40% of incubated drug) and this binding decreased to about 20% in the presence of NADPH. This reduction of the binding by NADPH might be due to reduction of the ortho-quinone to the catechol. This is supported by the observation that upon incubation of the ortho-quinone of VP-16-213 with NADPH cytochrome P-450 reductase, HPLC-analysis of the incubation mix-

Table 1. Effects of incubation conditions on in vitro covalent binding of ^3H -ortho-quinone and ^3H -VP-16-213 to rat liver microsomal proteins

Incubation conditions	% of drug covalently bound ^a
Ortho-quinone + NADPH	20.5 \pm 1.1
Ortho-quinone - NADPH	39.9 \pm 0.9 ^b
Ortho-quinone - NADPH + DPE	6.8 \pm 0.2 ^b
VP-16-213 + NADPH	2.7 \pm 0.3
VP-16-213 - NADPH	0.3 \pm 0.1 ^c
VP-16-213 + NADPH + ethylenediamine	1.6 \pm 0.1 ^c
VP-16-213 + NADPH + ascorbic acid	0.5 \pm 0.1 ^c

^a Mean \pm SD for four experiments.^b $P < 0.001$ when compared to ortho-quinone + NADPH.^c $P < 0.001$ when compared to VP-16-213 + NADPH.

ture revealed the immediate conversion of the ortho-quinone into a product with the same retention time as the synthetic catechol of VP-16-213. Mass spectrometric analysis of the isolated component showed peaks characteristic for the catechol structure ($m/z = 572, 368$). In contrast to the binding of the ortho-quinone, the covalent binding of the parent drug VP-16-213 was dependent on NADPH, but was about 15 times lower than that of the ortho-quinone in absence of NADPH. This indicates that VP-16-213 is in part converted to a metabolite responsible for the covalent binding of VP-16-213 and this metabolite might be the electrophilic ortho-quinone, which can react with nucleophilic microsomal components. Binding of VP-16-213 was diminished to the control level by ascorbic acid, which could be explained by reduction of the ortho-quinone to the catechol of VP-16-213. Ethylenediamine, a known ortho-quinone trapping agent [25], inhibited binding of VP-16-213 to microsomal proteins by about 40%, and the binding to microsomal proteins of the ortho-quinone itself was inhibited by 1,2-diphenylethylenediamine by 83%, again indicating that the ortho-quinone is the binding species.

One possible mechanism for the conversion of the catechol into the ortho-quinone is that the catechol is oxidized "spontaneously" by oxygen. To test this possibility, the catechol of VP-16-213 was incubated in 5×10^{-2} M phosphate buffer pH 7.4 at 37° for increasing periods of time under air. During this incubation, the formation of a component with the same retention time as that of the ortho-quinone was observed (Fig. 3A). If the catechol was incubated in an atmosphere of nitrogen, no formation of ortho-quinone was observed, but the formation of a component with longer retention time than that of the catechol (product x, Fig. 3B). If this incubation was continued in an atmosphere of air, again formation of the ortho-quinone took place and formation of products with short retention time (Fig. 3C). Separate long-term incubations of catechol and ortho-quinone at pH 7.4 and 37° resulted in the same HPLC-pattern of products with shorter retention time, suggesting the oxidation of catechol into ortho-quinone, followed by decomposition of the ortho-quinone (Fig. 3D). If the ortho-quinone was incubated at 37° in 5×10^{-2} M phosphate buffer pH 4, no conversion of the ortho-quinone was observed, indicating that the ortho-quinone is stable at pH

4 (Fig. 3E). However, upon incubation at 37° in 5×10^{-2} M phosphate buffer pH 8.6, a very fast conversion of the ortho-quinone took place to products with short retention time when compared to the incubation at pH 7.4 (Fig. 3F). In Fig. 4A, the chemical stability of the ortho-quinone is shown upon incubation at pH 4, 7.4 or 8.5: upon incubation at pH 8.5, the ortho-quinone was completely converted in about 25 min, whereas again upon incubation at pH 4.0 it was stable. At pH 7.4, an intermediate stability was observed. In comparison with the ortho-quinone, the catechol of VP-16-213 was also stable at pH 4.0, but proved to be much more stable at pH 7.4 (Fig. 4B). The pH-dependency of the stability of the ortho-quinone was confirmed by spectrophotometric experiments. The UV spectrum of the ortho-quinone showed an absorption maximum at 365 nm, which remained present upon incubation at pH 4.0, but disappeared upon incubation at pH 7.4 (Fig. 5).

A second mechanism by which ortho-quinone can be formed from catechol may be through oxidation of the catechol by superoxide anions formed by autooxidation of cytochrome P-450 analogous to the proposed oxidation of other catechols into ortho-quinones [26]. To test this hypothesis, a small amount of potassium superoxide was added to an aqueous solution of the catechol. A component was formed with the same retention time in HPLC as the ortho-quinone, which could be electrochemically reduced at -250 mV, but not—like the catechol—be electrochemically oxidized at +200 mV (data not shown).

The results in this section indicate that the catechol of VP-16-213, formed by *O*-demethylation, can be converted into the ortho-quinone both by spontaneous oxidation and oxidation by superoxide anions and that the ortho-quinone is the principal binding species to microsomal proteins.

(b) *Catechol and ortho-quinone of VP-16-213: binding to calf thymus DNA and inactivation of ΦX174 DNA*

Binding to calf thymus DNA. In order to establish whether the catechol and ortho-quinone are able to bind to DNA, incubations of ^3H -labelled catechol, ortho-quinone and VP-16-213 with calf thymus DNA were performed for 1 hr at 37° at pH 4.0 and 7.4, followed by determination of the amount of label

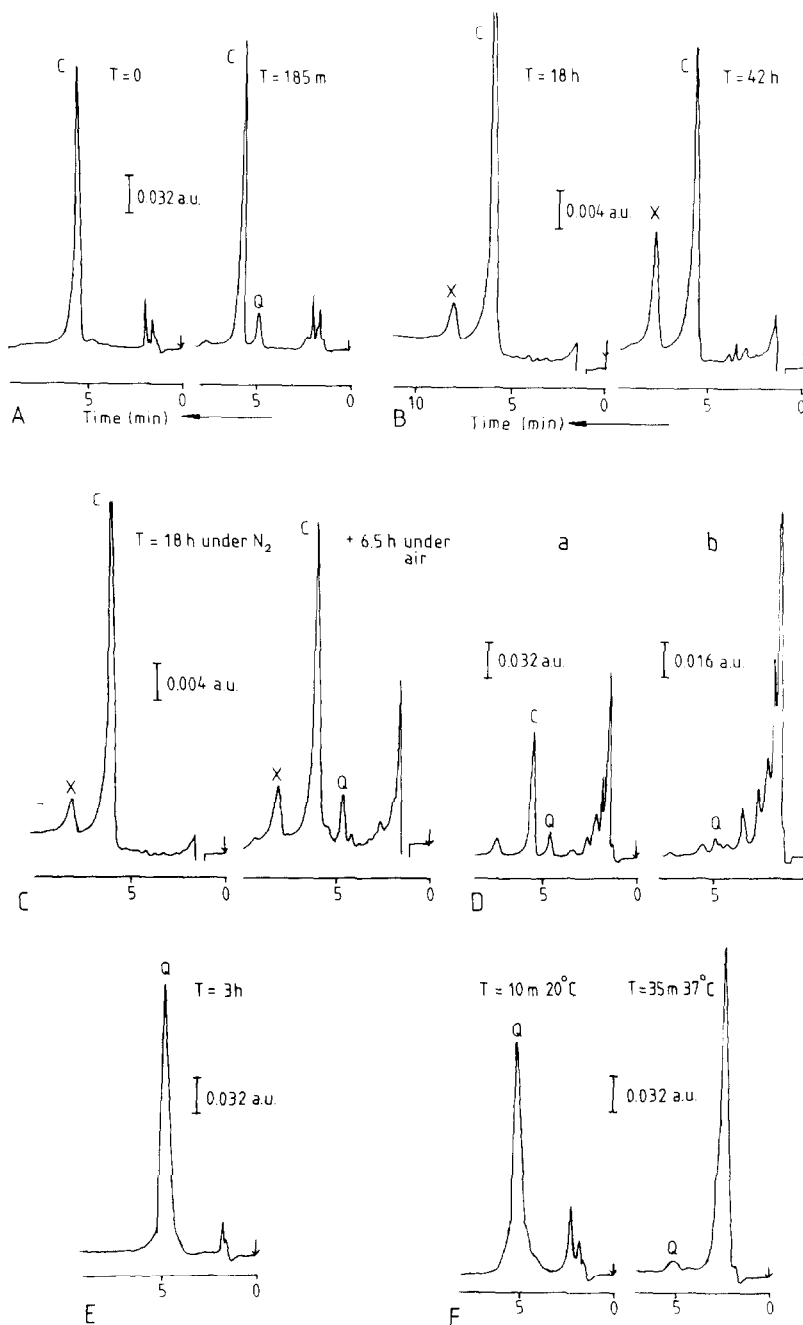


Fig. 3. HPLC-chromatograms of mixtures obtained after incubation of 1.8×10^{-4} M catechol (C) or ortho-quinone (Q) of VP-16-213 at 37°C . UV detection at 254 nm was used. The eluent used was methanol/water (50/50 v/v). m = min; h = hr. (A) Catechol in 5×10^{-2} M phosphate buffer pH 7.4 under air; (B) catechol in 5×10^{-2} M phosphate buffer pH 7.4 under N_2 ; (C) catechol in 5×10^{-2} M phosphate buffer pH 7.4 under N_2 for 18 hr, followed by incubation under air for 6.5 hr; (D) catechol (a) and ortho-quinone (b) for 18 and 43 hr, respectively, in 5×10^{-2} M phosphate buffer pH 7.4; (E) ortho-quinone in 5×10^{-2} M phosphate buffer pH 4.0; (F) ortho-quinone in 5×10^{-2} M phosphate buffer pH 8.6.

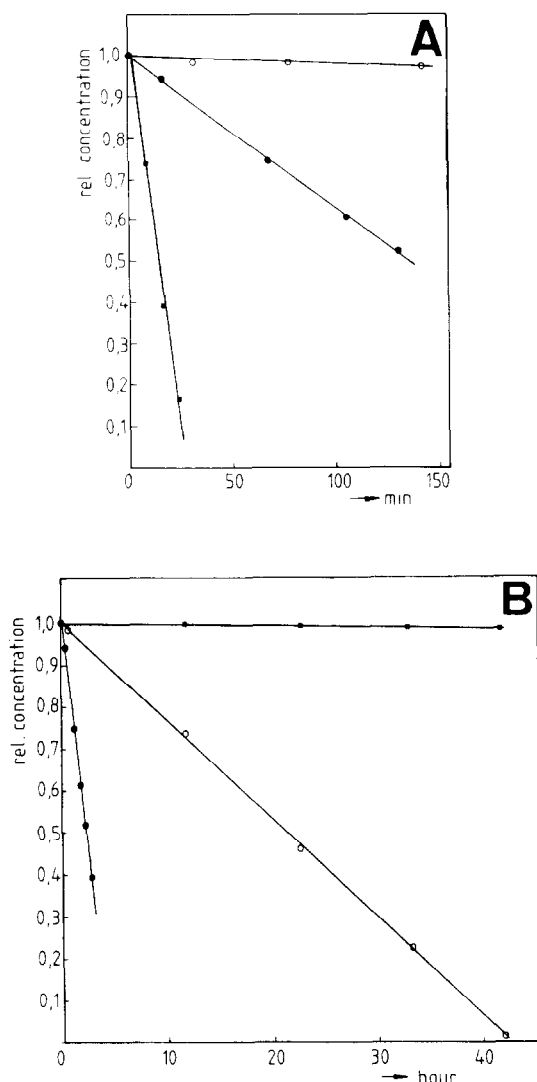


Fig. 4. (A) The stability of the ortho-quinone of VP-16-213 (1.8×10^{-4} M) in 5×10^{-2} M phosphate buffer of pH 4.0 (○), pH 7.4 (●) and pH 8.5 (■), as measured by HPLC-analysis. (B) The stability of the ortho-quinone of VP-16-213 (1.8×10^{-4} M) in 5×10^{-2} M phosphate buffer of pH 7.4 (●) and of the catechol of VP-16-213 (1.8×10^{-4} M) in 5×10^{-2} M phosphate buffer of pH 7.4 (○) or pH 4.0 (■), as measured by HPLC-analysis.

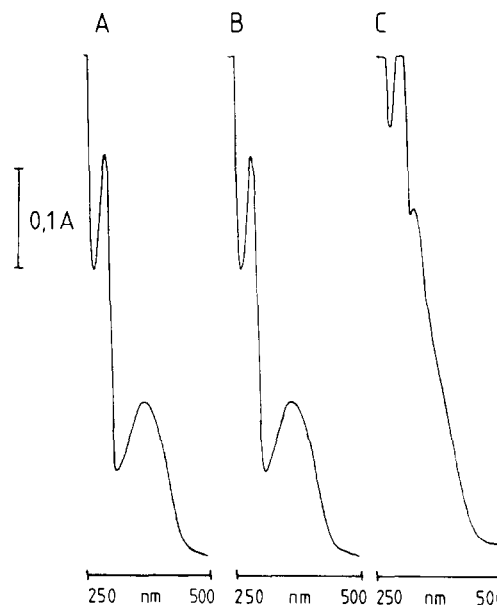


Fig. 5. Spectrophotometric experiments on the stability of the ortho-quinone of VP-16-213. UV spectrum of the ortho-quinone (1.8×10^{-4} M) after 0 (A) and 18 hr (B) of incubation at 37° in 5×10^{-2} M phosphate buffer pH 4.0; and after 0 (A) and 18 hr (C) of incubation at 37° in 5×10^{-2} M phosphate buffer 7.4.

bound to the DNA. Table 2 shows the amount of drug bound to DNA at the different pHs. At pH 4.0, catechol and ortho-quinone showed about 7 and 5 times more binding than VP-16-213. At pH 7.4, catechol and ortho-quinone showed about 9 and 7 times more binding to DNA than VP-16-213. Since catechol and ortho-quinone are stable upon incubation at pH 4.0, they must be the real binding species at that pH. The results indicate that catechol and ortho-quinone bind stronger to DNA than VP-16-213, the catechol is the strongest binding species of the two metabolites and the decomposition products of catechol and ortho-quinone formed at higher pH bind stronger to the DNA than catechol and ortho-quinone themselves. Ortho-quinone was found to bind about 2.5 times, and catechol about 8 times stronger to denatured calf thymus DNA than to double-stranded DNA.

Inactivation of ss and RF Φ X174 DNA by the catechol of VP-16-213. Incubation of ss and RF Φ X174 DNA at 37° and pH 7.4 under air with the catechol of VP-16-213 resulted in inactivation of

Table 2. Binding of VP-16-213, catechol and ortho-quinone to calf thymus DNA (pmol bound/ μ g DNA)^a

pH of incubation medium	VP-16-213	Catechol	Ortho-quinone
4.0	1.8 ± 0.1	12.4 ± 2.0	8.6 ± 1.3
7.4	2.7 ± 0.1^b	25.0 ± 2.7^c	18.9 ± 2.3^c

^a Results of two experiments.

^b $P < 0.01$ when compared to pH 4.0.

^c $P < 0.05$ when compared to pH 4.0.

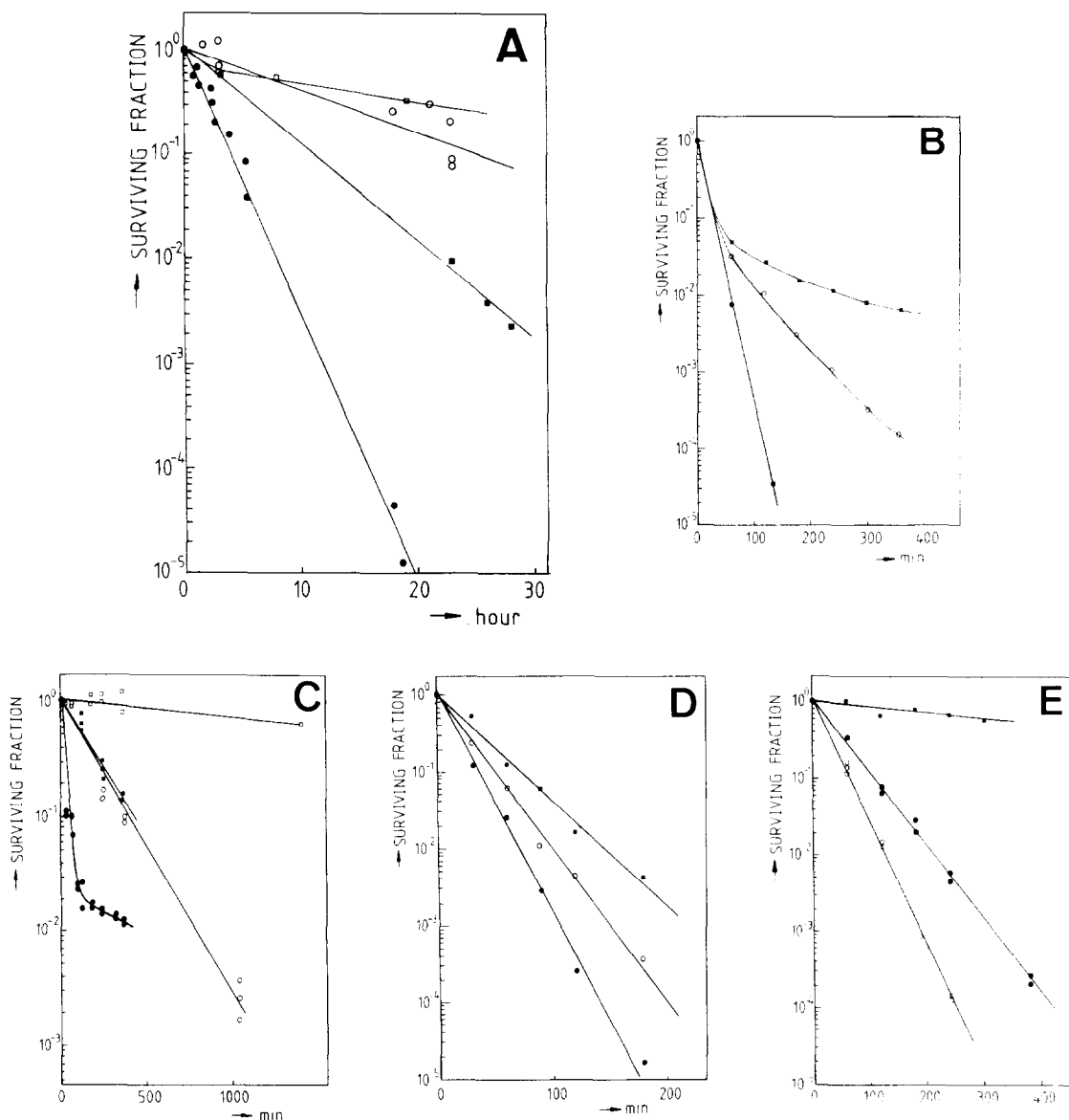


Fig. 6. (A) Survival curves of single-stranded (●) and double-stranded (○) Φ X174 DNA incubated at pH 7.4 with the catechol of VP-16-213 and for single-stranded Φ X174 DNA incubated at pH 7.4 with the ortho-quinone of VP-16-213 and NADPH cytochrome P-450 reductase, in absence (■) or presence (□) of DMSO. (B) Survival curves of single-stranded Φ X174 DNA incubated with the ortho-quinone of VP-16-213 at pH 4.0 (●), 5.0 (○) and 7.4 (■). (C) Survival curves of single-stranded Φ X174 DNA incubated with the ortho-quinone of VP-16-213 kept in phosphate buffer pH 7.4 for 0 (●), 6 (○) and 21 hr (■), and at pH 7.4 without ortho-quinone (□), respectively. (D) Survival curves of single-stranded Φ X174 DNA incubated at pH 4.0 with 1.8×10^{-4} M (●), 0.9×10^{-4} M (○) and 0.25×10^{-4} M (■) ortho-quinone of VP-16-213. (E) Survival curves of double-stranded Φ X174 DNA incubated at pH 4.0 with the ortho-quinone of VP-16-213 using wild type (●), *uvrA*⁻ (○) or *uvrC*⁻ (□) *E. coli* in the spheroplast-assay, and at pH 4.0 without ortho-quinone (■), respectively.

DNA; a typical example is shown in Fig. 6A. The inactivation of ss Φ X174 DNA had a mean T_{37} -value—incubation time resulting in 63% DNA-inactivation—of 96 ± 3 min. RF Φ X174 DNA was inactivated by the catechol of VP-16-213 with a mean T_{37} -value of 640 ± 76 min. Incubation of ss Φ X174 DNA with the ortho-quinone of VP-16-213 and NADPH cytochrome P-450 reductase at pH 7.4,

which immediately converts the ortho-quinone into the catechol (see a), resulted in inactivation of the DNA with a mean T_{37} -value of 454 ± 21 min. If the ortho-quinone was added to the incubation mixture by dissolution in 0.5% v/v DMSO, hardly any inactivation was observed (see Fig. 6A).

Inactivation of ss and RF Φ X174 DNA by the ortho-quinone of VP-16-213. The inactivation of ss

DNA upon incubation with the electrochemically prepared ortho-quinone was found to be dependent on the pH, corresponding with the pH-dependent chemical stability of the ortho-quinone as described in the previous section (Fig. 4A). At higher pHs less inactivation of DNA was found. The survival curves of ss Φ X174 DNA incubated with the ortho-quinone at pH 4.0, 5.0 and 7.4 are shown in Fig. 6B. Only after incubation of ss Φ X174 DNA at pH 4.0, at which pH the ortho-quinone is stable (Fig. 4A), an exponential survival curve was obtained. The T_{37} -value for inactivation of ss Φ X174 DNA at pH 4.0 at a concentration of 1.8×10^{-4} M ortho-quinone was 24 ± 1 min. At pH 5.0 and 7.4 the inactivation showed a biphasic pattern: a fast initial phase of inactivation was followed by a slower phase, suggesting that the aqueous decomposition product(s) of the ortho-quinone also inactivated DNA, but to a lower extent than the ortho-quinone itself. To test this idea, solutions of the ortho-quinone were kept at pH 7.4 for 6 and 21 hours and incubated with ss Φ X174 DNA. These solutions with the aqueous decomposition products of the ortho-quinone inactivated the DNA with a T_{37} -value of 175 ± 6 and 183 ± 10 min, respectively (Fig. 6C), in reasonable agreement with the T_{37} -value of the second phase of inactivation at pH 7.4 found in Fig. 6B. The inactivation of ss and RF Φ X174 DNA by the ortho-quinone at pH 4.0 was found to be concentration-dependent: increasing inactivation of DNA was found at increasing concentration of the ortho-quinone. Figure 6D shows the survival curves of ss Φ X174 DNA incubated at pH 4.0 with different concentrations of the ortho-quinone. A linear relationship was observed between increasing concentration ortho-quinone and decreasing T_{37} -value. Incubation with the ortho-quinone at pH 4.0 led to inactivation of RF Φ X174 DNA with a rate twice as low as the rate of inactivation of ss Φ X174 DNA: the T_{37} -value for inactivation by 1.8×10^{-4} M ortho-quinone was 49 ± 2 min (Fig. 6E). When using excision-repair deficient *E. coli* mutants (*uvrA*⁻ or *uvrC*⁻) in the spheroplast-assay, a higher inactivation of RF Φ X174 DNA was found: $T_{37} = 29$ min for *uvrA*⁻ *E. coli* (Fig. 6E), indicating that a part of the DNA damage introduced by the incubation with ortho-quinone was removed by excision repair. No

difference in the rate of inactivation of DNA was found when using either *uvrA*⁻ or *uvrC*⁻ *E. coli* in the spheroplast-assay.

Analysis of DNA damage after incubation of the ortho-quinone with ss Φ X174 DNA at pH 4.0 by sucrose gradient centrifugation under neutral and alkaline conditions revealed that no DNA-breakage had occurred.

DISCUSSION

The results presented in this study give further support for the hypothesis that oxidative biotransformation of the E-ring of VP-16-213—in particular to the catechol and ortho-quinone metabolites—may play an important role in DNA inactivation. The formation of the ortho-quinone on peroxidation of VP-16-213 by horseradish peroxidase has been reported by Haim *et al.* [12]. The intermediate formed in this peroxidation was the phenoxy radical of VP-16-213 [12, 27]. Recently, it has been reported that VP-16-213 was *O*-demethylated by cytochrome P-450 to the catechol metabolite [5, 10, 11]. The present findings indicate that the ortho-quinone can be formed as a secondary metabolite of VP-16-213 by oxidation of the catechol. If the catechol was incubated in buffer under air, formation of the ortho-quinone and its decomposition products occurred, and not on incubation under nitrogen. The results of experiments on covalent binding of VP-16-213 and its ortho-quinone to rat liver microsomes indicated the formation of the ortho-quinone in microsomal incubations of VP-16-213, probably by oxidation of the catechol formed by *O*-demethylation of VP-16-213, and that the metabolite responsible for the covalent binding of VP-16-213 to microsomal proteins is the ortho-quinone. The latter possibility was recently suggested by Haim *et al.* [10]. On incubation with microsomes, the ortho-quinone can be formed by oxidation of the catechol by molecular oxygen, or by oxidation of the catechol by superoxide anions formed by auto-oxidation of cytochrome P-450. The production of the quinaxaline of the ortho-quinone of VP-16-213 on incubation of VP-16-213 with microsomes in the

Table 3. T_{37} -values for inactivation of ss and RF Φ X174 DNA by ortho-quinone and catechol of VP-16-213 in different test systems

Test system ^a	T_{37} (min) ^b
Catechol/pH 7.4/ss DNA	96 ± 3
Catechol/pH 7.4/RF DNA	640 ± 76
<i>O</i> -Quinone/pH 7.4/ss DNA/P-450 reductase	454 ± 21
<i>O</i> -Quinone/pH 7.4/ss DNA/P-450 reductase/DMSO	1987 ± 124
<i>O</i> -Quinone/pH 4.0/ss DNA	24 ± 1
<i>O</i> -Quinone 6 hours at pH 7.4/ss DNA	175 ± 6
<i>O</i> -Quinone 21 hours at pH 7.4/ss DNA	183 ± 10
<i>O</i> -Quinone/pH 4.0/RF DNA	49 ± 2
<i>O</i> -Quinone/pH 4.0/RF DNA/ <i>uvrA</i> ⁻ <i>E. coli</i>	29 ± 2

^a Wild type *E. coli* was used in the spheroplast-assay except where otherwise indicated. The concentration of the catechol was 2.2×10^{-4} M and of the ortho-quinone 1.8×10^{-4} M.

^b Results of 3–4 experiments.

presence of DPE is also suggestive for the formation of the ortho-quinone.

Inactivation of DNA by catechol and ortho-quinone was investigated in detail, using Φ X174 DNA as model system. Table 3 summarizes the T_{37} -values for inactivation of ss and RF Φ X174 DNA under different conditions. The rate of inactivation of ss DNA by the catechol is about 6 times higher than the rate of inactivation of RF DNA. The inactivation of ss Φ X174 DNA by the ortho-quinone incubated with NADPH cytochrome P-450 reductase is probably caused by the catechol formed by reduction of the ortho-quinone. The fact that this system inactivated ss Φ X174 DNA with a T_{37} -value about five times as low as the value for inactivation by the catechol might be explained by binding of part of the ortho-quinone to the reductase without conversion to the catechol. The inactivation of ss Φ X174 DNA by ortho-quinone/NADPH cytochrome P-450 reductase was found to be inhibited by DMSO. This inhibition by DMSO can be explained by trapping of a free radical, e.g. the semi-quinone radical, which will be the intermediate in the conversion of the ortho-quinone into the catechol [28]. This is supported by the observation that DMSO had no effect on the inactivation of DNA by the ortho-quinone itself. Inactivation of ss Φ X174 DNA by the ortho-quinone was found to be pH-dependent: less inactivation was observed at higher pH. This can be explained by the low stability of the ortho-quinone at high pH. However, the product formed from the ortho-quinone at pH 7.4 also inactivates DNA, but to a lower extent than the ortho-quinone itself. The rate of inactivation of ss DNA by the ortho-quinone is about two times higher than the rate of inactivation of RF DNA. Since the catechol is converted upon incubation under air at pH 7.4 into the ortho-quinone, the possibility exists that the inactivation of DNA by the catechol is due to the formation of the ortho-quinone. However, since a difference of a factor 2 exists between the T_{37} -values for inactivation of ss and RF DNA by the ortho-quinone and a difference of a factor 7 between the T_{37} -values for inactivation by the catechol, it is not likely that the inactivation of DNA by the catechol is due to formation of the ortho-quinone.

From the experiments on binding of VP-16-213, catechol and ortho-quinone to calf thymus DNA, it can be concluded that the catechol binds more strongly to DNA than the ortho-quinone, and that the decomposition products bind stronger than catechol and ortho-quinone themselves. The higher sensitivity of ss Φ X174 DNA than of RF DNA to ortho-quinone and catechol could possibly be explained by the higher amount of adducts formed, since ortho-quinone binds about 2.5 times more strongly and catechol about 8 times more strongly to ss DNA than to RF DNA, and the inactivation of ss DNA by ortho-quinone and catechol is a factor of 2 (ortho-quinone) and 7 (catechol) higher than the inactivation of RF DNA. However, since the T_{37} -values for inactivation of both ss and RF DNA by the catechol are much higher than the T_{37} -values for inactivation by the ortho-quinone, it could be suggested that most of the binding products of the catechol and the decomposition products are not

relevant for inactivation of DNA or are much better repaired than the products of the ortho-quinone. A part of the difference in sensitivity to the ortho-quinone between ss and RF DNA can be explained by excision repair of the damage in RF DNA, since RF Φ X174 DNA is more extensively inactivated by the ortho-quinone at pH 4 if excision repair deficient *E. coli* mutants (*uvrA*⁻) are used. This indicates that: (a) adduct-formation takes place involving local distortions of the DNA-helix; (b) there are at least two types of adducts: inactivating adducts which are not repaired by excision repair and non-lethal adducts which are repaired. It is possible that some adducts formed intercalate after binding or give insertion denaturation. The absence of DNA breakage observed after neutral and alkaline sucrose gradient centrifugation of ss Φ X174 DNA incubated with the ortho-quinone shows that the formation of breaks or alkali-labile sites does not play a role in the inactivation of ss Φ X174 DNA by the ortho-quinone.

The finding that the catechol is adducted in higher amount to DNA than the ortho-quinone, while for binding to microsomal proteins the reverse holds true, could possibly be explained by the suggestion that binding of ortho-quinone to proteins and DNA is due to a covalent attachment of the keto functions of the ortho-quinone to SH-groups of proteins and nitrogen atoms of DNA bases, while for the binding of catechol not a covalent attachment but strong hydrogen bonding occurs, which would contribute substantially to binding to DNA but not to binding to proteins. This could also be supportive for the suggestion that most of the catechol adducts are not relevant for inactivation of DNA or are much better repaired than the ortho-quinone adducts.

Since after incubation of VP-16-213 with rat liver microsomes for 1 hr at 37°, 4–5% of VP-16-213 is converted into catechol, the formation of the reactive metabolites catechol and ortho-quinone could be relevant for the process of DNA inactivation by VP-16-213. Although the activity of VP-16-213 on topoisomerase II has been demonstrated *in vitro* in the absence of metabolism, the observation that the 4'-OH group in the E-ring of VP-16-213 is necessary for the DNA-damaging effect is also important when discussing the mechanism of inactivation of DNA by VP-16-213. In view of the formation of the reactive metabolites catechol and ortho-quinone in the E-ring of VP-16-213, we suggest testing catechol and ortho-quinone of VP-16-213 for their ability to inhibit topoisomerase II. The possibility exists that both the topoisomerase and the metabolic activation hypothesis are important for the mechanism of action of VP-16-213.

In summary, we found evidence for the formation of the ortho-quinone as a metabolite of VP-16-213 with the catechol of VP-16-213 as intermediate. The formation of more polar autooxidation products has been shown on incubation of both the catechol and the ortho-quinone. Catechol and ortho-quinone of VP-16-213 bind more strongly than VP-16-213 to calf thymus DNA. In contrast to VP-16-213 [4, 5], the catechol as well as the ortho-quinone inactivated Φ X174 DNA. Adduct-formation probably plays a role in the inactivation of DNA by the ortho-quinone. These findings suggest that the cytotoxicity

of VP-16-213 could be based upon metabolic activation of the E-ring of VP-16-213 to products which can cause DNA damage.

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