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EFFECTS OF OXYGEN RADICAL SCAVENGERS ON THE INACTIVATION OF SS ΦX174 DNA BY THE SEMI-QUINONE FREE RADICAL OF THE ANTITUMOR AGENT ETOPOSIDE

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We have studied the effects of oxygen radical scavengers on the inactivation of ss ΦX174 DNA by the semi-quinone free radical of the antitumor agent etoposide (VP 16-213), which was generated from the ortho-quinone of etoposide at pH $\geq$ 7.4. A semi-quinone free radical of etoposide is thought to play a role in the inactivation of ss ΦX174 DNA by its precursors 3',4'-*ortho-quinone* and 3',4'-*ortho-dihydroxy*-derivative. The possible role of oxygen radicals formed secondary to semi-quinone formation in the inactivation of DNA by the semi-quinone free radical was investigated using the hydroxyl radical scavengers t-butanol and DMSO, the spin trap DMPO, the enzymes catalase and superoxide dismutase, the iron chelator EDTA and potassium superoxide. Hydroxyl radicals seem not important in the process of inactivation of DNA by the semi-quinone free radical, since t-butanol, DMSO, catalase and EDTA had no inhibitory effect on DNA inactivation. The spin trapping agent DMPO strongly inhibited DNA inactivation and semi-quinone formation from the ortho-quinone of etoposide at pH $\geq$ 7.4 with the concomitant formation of a DMPO-OH adduct. This adduct probably did not arise from OH· trapping but from trapping of O_2^{-}· . DMSO increased both the semi-quinone formation from and the DNA inactivation by the ortho-quinone of etoposide at pH $\geq$ 7.4. Potassium superoxide also stimulated ΦX174 DNA inactivation by the ortho-quinone at pH $\leq$ 7. From the present study, it is also concluded that superoxide anion radicals probably play an important role in the formation of the semi-quinone free radical from the orthoquinone of etoposide, thus indirectly influencing DNA inactivation.

KEY WORDS: Etoposide, semi-quinone, oxygen radical scavengers, DNA inactivation.

INTRODUCTION

Etoposide (NSC 141540, VP 16-213, Figure 1) is an important antineoplastic agent used against several types of tumors including testicular and small cell lung cancers, lymphoma, leukemia and Kaposi's sarcoma.[1] The cytotoxicity of etoposide may

Abbreviations used: ESR, electron spin resonance; HPLC, high performance liquid chromatography; ss, single-stranded; SOD, superoxide dismutase; CAT, catalase; DMSO, dimethyl sulfoxide; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; EDTA, ethylenediamine tetraacetic acid; KO_2, potassium superoxide.

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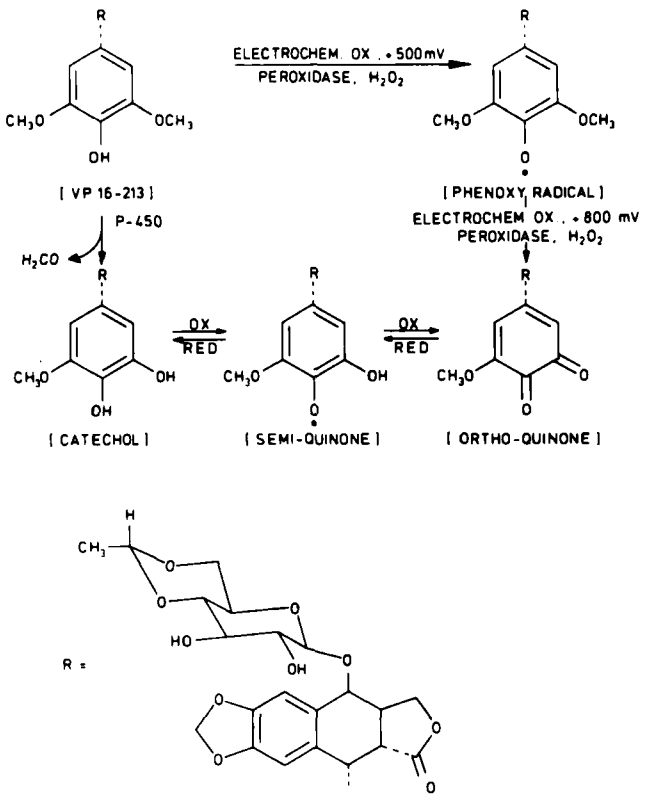

FIGURE 1 Metabolic conversions of the dimethoxyphenol ring of VP-16-213.

depend on a dual mechanism of DNA deactivation.[2] The nuclear enzyme DNA topoisomerase II probably plays a predominant role in mediating etoposide-induced DNA strand scission.[3-6] Etoposide interferes with the breakage-reunion reaction of DNA topoisomerase II by stablilizing a cleavable complex. Besides by disturbing the DNA topoisomerase II reaction, however, etoposide could also exert its cytotoxic effects by bioactivation to products which can cause DNA damage. The presence of the 4′-OH group in the dimethoxyphenol ring (the E-ring) of etoposide was found to be a structural prerequisite for the DNA damaging and cytotoxic effects of etoposide. Purified DNA is not damaged by the parent drug.[7,8] For this reason, we previously studied possible metabolic conversions of the E-ring of etoposide. O-demethylation of the E-ring of etoposide by rat and mouse liver microsomes and by purified cytochrome P-450 has been reported by us,[9-11] by Sinha *et al.*[12] and Haim *et al.*[13] The product of O-demethylation was identified as the 3′,4′-dihydroxy derivative or catechol of etoposide;[11,13] (Figure 1). The catechol can subsequently be oxidized to the ortho-quinone of etoposide,[14] a metabolite which is also produced by a peroxidative activation of etoposide.[15] Both the ortho-quinone and the catechol of etoposide bind strongly to purified calf thymus DNA *in vitro* and inactivate biologically active single- and double-stranded ΦX174 DNA, in contrast to the parent compound etoposide.[11,14,16] These findings gave support to the hypothesis that the antitumor activity

of etoposide may also be based on the metabolic activation of etoposide to metabolites which can inactivate DNA.

Earlier we described the formation of a semi-quinone free radical of etoposide,[17] (Figure 1). It has been suggested that this species may play a role in the inactivation of DNA by the catechol and ortho-quinone of etoposide.[17,18] In a recent study, Kalyanaraman *et al.* reported the formation of both primary and secondary phenoxyl and semi-quinone free radicals from etoposide.[19] It was suggested that none of these free radicals of etoposide react with DNA at a detectable rate. However, our recent results,[20] as well as the results of the present study show that the (primary) semi-quinone free radical of etoposide strongly inactivates single-stranded ΦX174 DNA, although this species does not affect double-stranded ΦX174 DNA.

The formation of oxygen radicals during redox-cycling of quinoid anticancer agents is well documented.[21] The possibility exists that oxygen radicals also play a role in the inactivation of DNA by the semi-quinone free radical of etoposide. Indications for the formation of oxygen radicals by redox-cycling of the ortho-quinone of etoposide during reduction by NADPH cytochrome P-450 reductase were obtained previously.[17] The aim of the present study was to investigate the possible role of oxygen radicals in the inactivation of ss ΦX174 DNA by the semi-quinone free radical of etoposide.

MATERIALS AND METHODS

Drugs and chemicals

Etoposide was a gift from the Bristol Myers Company (Syracuse, NY, U.S.A.). The ortho-quinone of etoposide was synthesized by controlled potential electrolysis of etoposide at a Pt-gauze electrode.[22] The 3′,4′-dihydroxy-derivative of etoposide (the catechol) was synthesized from the ortho-quinone of etoposide by reduction with ascorbic acid.[11] Superoxide dismutase (SOD) and catalase were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Potassium superoxide (KO_2) and 5,5-dimethyl-l-pyrroline-N-oxide (DMPO) were purchased from Aldrich Chemical Company, Milwaukee, WIS, U.S.A. All other chemicals used were obtained from the laboratory stock and were reagent grade.

Incubations with single-stranded (ss) ΦX174 DNA

Single-stranded ΦX174 DNA was isolated from wild-type ΦX174 DNA bacteriophage according to Blok *et al.*[23] Solutions of ss ΦX174 DNA (12.5 ng/ml) in 5×10^{-2} M potassium phosphate pH 7.4 and pH 9, respectively, were incubated at 37°C for increasing periods of time under aerobic conditions with the ortho-quinone of etoposide (4.4×10^{-4} M) and (a) 10–30 v/v% DMSO, (b) 50–300 mM DMPO, (c) 30% DMSO + 300 mM DMPO, (d) 100 μg/ml SOD, (e) 1000–4000 U/ml catalase, (f) 0.1–1 M t-butanol, (g) 25 mM EDTA and (h) 5 mM KO_2 + 25 mM EDTA; in a total volume of 400 μl. The incubations (h) were also carried out in 5×10^{-2} M potassium phosphate pH 4 and pH 6.5. At several time intervals of incubation, samples of 20 μl were taken and diluted 50-fold with ice-cold 0.025 M *Tris*-HCl pH 8.2 to stop the reaction. Changes in biological activity of ΦX174 DNA were determined by measuring the bacteriophage production after transfection of the DNA to *E. coli* spheroplasts, as described in detail before.[14]

High performance liquid chromatography (HPLC)

HPLC was performed using a Waters 6000A Solvent Delivery System in combination with a Uvikon 740 LC Spectrometer (Kontron, Zürich, Switzerland) operating at $\lambda = 280$ nm and a CP-Microspher 3 μm C18 column (100 × 4.6 mm), at a flow rate of 0.5 ml/min. The mobile phase consisted of methanol/8.3×10^{-2} M Potassium phosphate pH 4 (40/60, v/v%). The incubations described in the paragraph "Incubations with ss ΦX174 DNA" were repeated in the absence of DNA and at several time intervals, 50 μl-samples were taken, immediately chilled on ice and analyzed by HPLC.

Electron spin resonance spectroscopy (ESR)

ESR spectra were recorded at room temperature on a Bruker ESP-300 spectrometer with an ESP 1600 data processor, equipped with an ER 4102 ST standard rectangular cavity. The modulation frequency of the spectrometer was 100 kHz. The incubations described in the paragraph "Incubations with ss ΦX174 DNA" were repeated in the absence of DNA, in a total volume of 500 μl, and at several time intervals subjected to ESR analysis. In addition, incubations at 37°C of the ortho-quinone of etoposide (4.4×10^{-4} M) in 5×10^{-2} M potassium phosphate pH 7.4 and pH 9, respectively, were carried out in the presence of (a) 300 mM DMPO and 20% ethanol, (b) 300 mM DMPO, 30% DMSO and 20% ethanol, (c) 300 mM DMPO and 25 mM EDTA and (d) 150 mM DMPO and 4000 U/ml catalase. At several time intervals, the incubation mixtures were subjected to ESR analysis.

The instrumental conditions were as follows: modulation amplitude, 0.2 G; reveiver gain, 3.2×10^5; conversion time, 10.24 msec; time constant, 2.56 msec; resolution of field axis, 4096; sweep time 41.94 sec; number of scans, 20; filter mode: polynomial filter, filter width 31; power, 50 mW; magnetic field, 3480 ± 10 G. For the incubations including DMPO, the magnetic field was 3480 ± 30 G.

RESULTS

The effects of addition of several radical scavengers/spin traps and enzymes on the inactivation of ss ΦX174 DNA by the semi-quinone free radical of etoposide generated from the corresponding ortho-quinone, and the analyses by ESR and HPLC of the incubation mixtures, will be discussed.

DMSO

The effect of DMSO was studies because of its ability to scavenge hydroxyl radicals.[24] Upon incubation of the ortho-quinone of etoposide with ss ΦX174 DNA at pH 7.4 in the presence of increasing concentrations of DMSO, an increasing rate of DNA inactivation was observed (Figure 2). The increased inactivation of DNA in the presence of DMSO was even higher at pH 9 (results not shown). ESR analysis showed that this was accompanied by an increasing intensity of the ESR signal (Figure 3). Both the intensity and the hyperfine splitting of the ESR signal changed with increasing concentration of DMSO. The HPLC analyses of the incubation mixtures revealed a faster decrease in ortho-quinone concentration with increasing concentrations of DMSO. Figure 4 shows the time course of the E.S.R. signal at pH 7.4 and 9, without

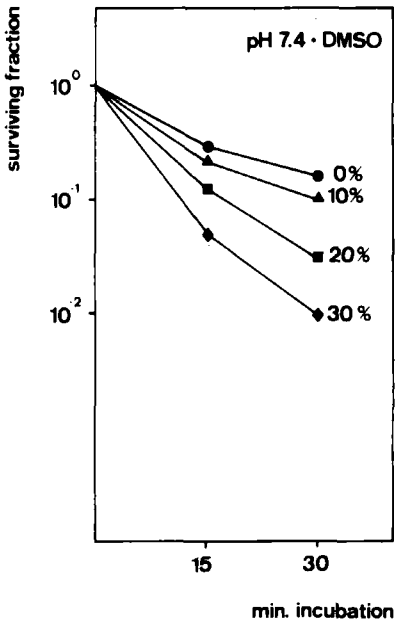

FIGURE 2 Survival curves of ss ΦX174 DNA incubated with the ortho-quinone of etoposide at pH 7.4 without DMSO (●) and in the presence of 10 v/v% (▲), 20 v/v% (■) and 30 v/v% (◆) DMSO.

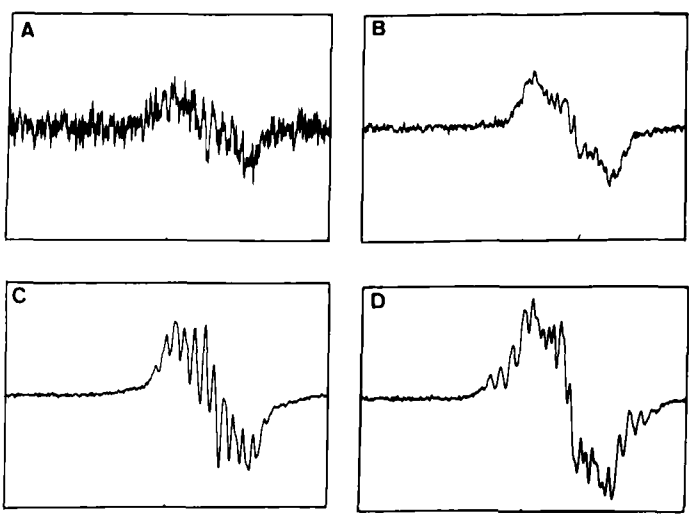

FIGURE 3 ESR signals of the semi-quinone free radical of etoposide formed during incubation of the ortho-quinone for 15 min at pH 7.4 without DMSO (A) and in the presence of 30% DMSO (B), and for 5 min at pH 9 without DMSO (C) and in the presence of 30% DMSO (D).

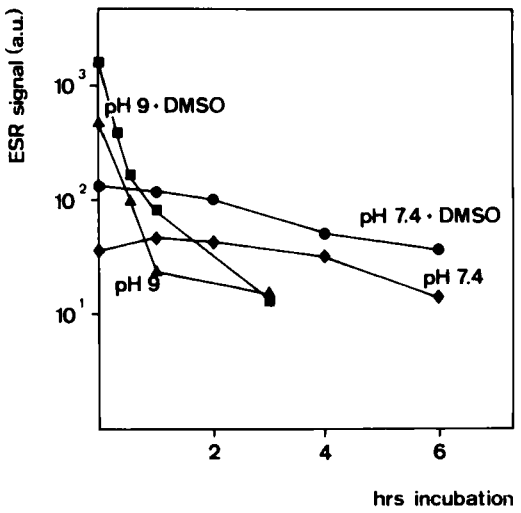

FIGURE 4 Relative intensity and decay of the ESR signal of the semi-quinone free radical of etoposide formed during incubation of the ortho-quinone at pH 7.4 without DMSO (◆) and in the presence of 30% DMSO (●), and at pH 9 without DMSO (▲) and in the presence of 30% DMSO (■).

DMSO and in the presence of 30% DMSO. As previously observed,[20] the free radical was present at a higher level in the incubation mixtures at pH 9 than in the incubation mixtures at pH 7.4, but its decay was faster at pH 9. In the presene of 30% DMSO, the intensity of the E.S.R. signal increased both at pH 7.4 and pH 9. Figure 5 shows the relationship between the $1/T_{37}$ value (a measure for the inactivation of DNA) obtained from the survival curves of ΦX174 DNA incubated with the ortho-quinone

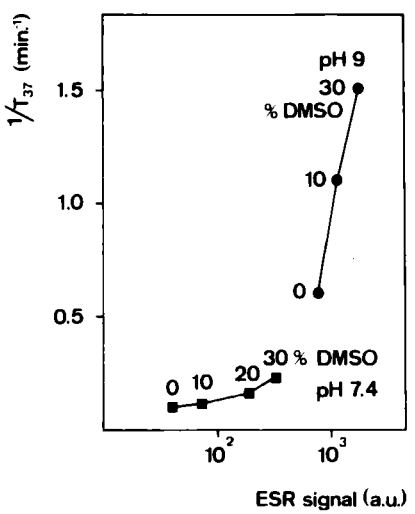

FIGURE 5 Correlation between DNA inactivation ($1/T_{37}$ value) and semi-quinone free radical formation during incubation of the ortho-quinone of etoposide at pH 7.4 and pH 9 in the presence of increasing concentration DMSO.

at pH 7.4 and pH 9 in the presence of different concentrations of DMSO, and the intensity of the semi-quinone free radical signal, indicating that an increase in the amount of semi-quinone free radical is accompanied by an increased rate of DNA inactivation.

DMPO

Addition of the spin trapping agent DMPO in the concentration range 50–300 mM to incubation mixtures of the ortho-quinone and ss ΦX174 DNA at pH 7.4 and 9 led to a strong inhibition of inactivation of DNA in a concentration-dependent manner. Figure 6 shows the survival curves of the incubation mixtures at pH 9. ESR analysis of the incubation mixtures showed that after 10 min incubation of the ortho-quinone at pH 9 in the presence of DMPO, the intensity of the semi-quinone signal decreased with the concomitant formation of a DMPO–OH adduct ($A_N = A_H^\beta = 14.9$ G; Figure 7A, B). Besides peaks of a DMPO–OH adduct (peaks a), three different peaks in between the signals of the DMPO–OH adduct were present (peaks b), one of which coincided with the peaks due to the semi-quinone. Peaks a and b increased in intensity with increasing concentration of DMPO. In incubations of the ortho-quinone at pH 7.4 the same phenomenon was observed (Figure 7C, D). After 1 hr of incubation of the ortho-quinone at pH 7.4 in the presence of 300 mM DMPO, the semi-quinone

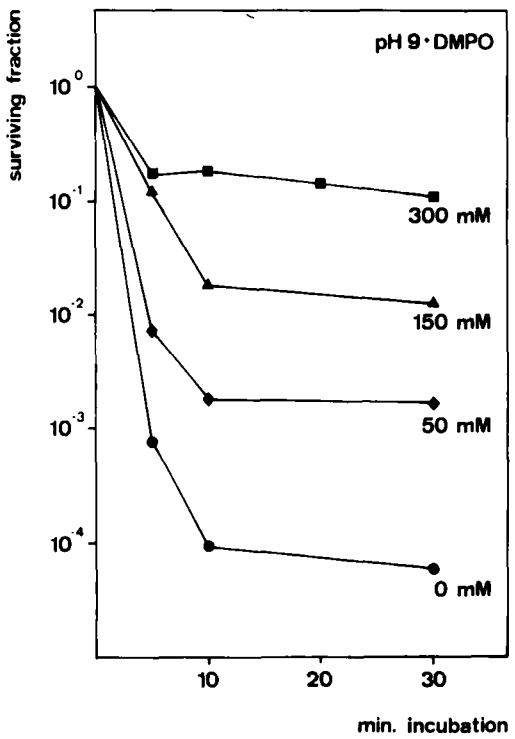

FIGURE 6 Survival curves of ss ΦX174 DNA incubated with the ortho-quinone of etoposide at pH 9 without DMPO (●) and in the presence of 50 mM (◆), 150 mM (▲) and 300 mM (■) DMPO.

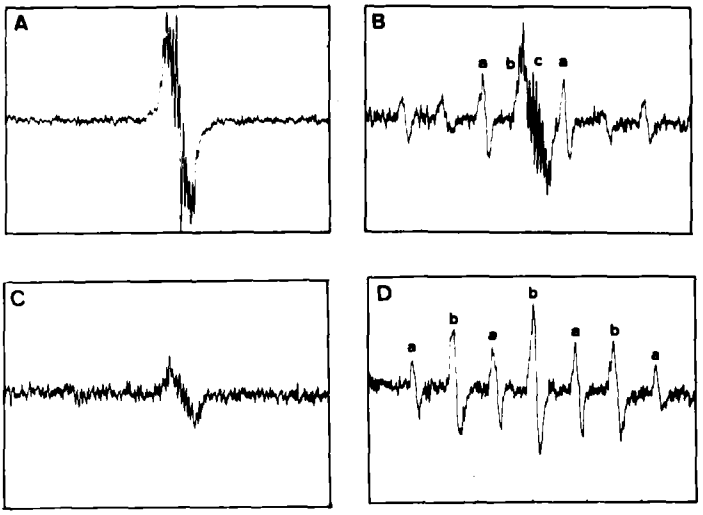

FIGURE 7 ESR signals of the incubation of the ortho-quinone of etoposide for 10 min at pH 9 without DMPO (A) and in the presence of 300 mM DMPO (B), and for 1 hour at pH 7.4 without DMPO (C) and in the presence of 300 mM DMPO (D). Peaks a are the peaks of the DMPO–OH signal.

signals had completely disappeared, while in the control incubation they were still present. The HPLC analysis of the incubation mixtures of ortho-quinone and DMPO showed at pH 9 almost immediate disappearance of the ortho-quinone with extenseive accumulation of the catechol of etoposide; at pH 7.4 the ortho-quinone had disappeared only after 2 hours with extensive formation of the corresponding catechol.

Since addition of DMSO and DMPO to the incubations of ortho-quinone at pH 7.4 and pH 9 had opposite effects on the inactivation of DNA, the effect of the combination of DMSO (30%) and DMPO (300 mM) was also investigated. This resulted in the same inhibitory effect on DNA inactivation as observed with DMPO alone. Figure 8 shows the survival curves of the incubation mixtures at pH 9. ESR analysis showed that in the presence of 300 mM DMPO and 30% DMSO, the semi-quinone signal was considerably decreased with accompanying formation of a DMPO–OH adduct (Figure 9).

The effects of several other incubation conditions, which would inhibit the direct trapping of OH· radicals by DMPO, were also investigated. Including 20% ethanol or 25 mM EDTA in the incubation mixture of the ortho-quinone in the presence of 300 mM DMPO had no effect on the DMPO–OH signal. 20% Ethanol also had no effect on the signal measured in the incubation mixture of the ortho-quinone with DMSO and DMPO (data not shown). These results indicate that in the formation of the DMPO–OH adduct, OH· radicals are not involved.

SOD

In order to investigate the role of superoxide anion radicals, the effect of SOD on DNA inactivation and semi-quinone formation was studied. Addition of 100 μg/ml SOD to incubation mixtures of ortho-quinone with ss ΦX174 DNA led to a small inhibitory effect on the inactivation of DNA at pH 9, while at pH 7.4 no effect was

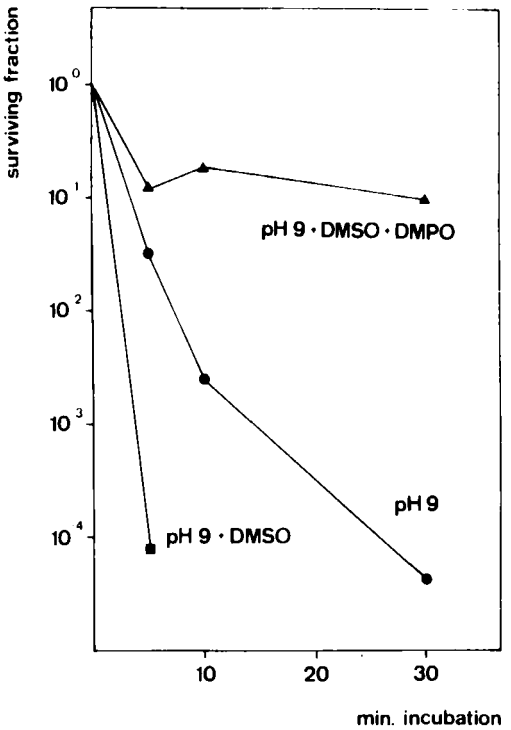

FIGURE 8 Survival curves of ss ΦX174 DNA incubated with the ortho-quinone of etoposide at pH 9, alone (●) and in the presence of 30% DMSO (■) and 30% DMSO + 300 mM DMPO (▲).

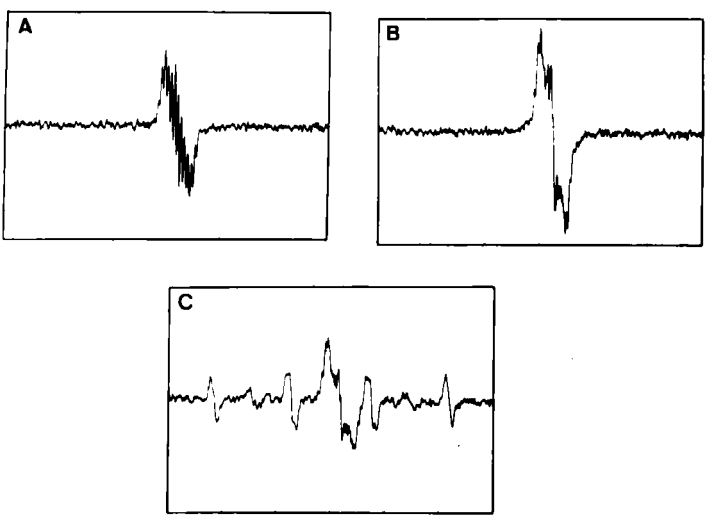

FIGURE 9 ESR signals of the incubation of the ortho-quinone of etoposide for 10 min at pH 9, alone (A) and in the presence of 30% DMSO (B) and 30% DMSO + 300 mM DMPO (C).

observed. ESR analysis showed a small decreasing effect of 100 μg/ml SOD on the ESR signal at pH 9, but not at pH 7.4. HPLC analysis revealed no significant effect of SOD on the rates of disappearance of the ortho-quinone at pH 7.4 nor at pH 9.

t-butanol

With the aim to investigate further the possible involvement of OH· radicals in the inactivation of ss ΦX174 DNA by the semi-quinone, t-butanol – a scavenger of OH· radicals[25] – was added to the incubation mixtures. 1 M t-butanol had no inhibitory effect on the DNA inactivation at pH 7.4 and pH 9.

ESR and HPLC analysis also showed neither effects on the semi-quinone free radical signal nor on the rate of disappearance of the ortho-quinone, in incubations at pH 7.4 and pH 9.

Catalase

To investigate a possible role of H_2O_2 on DNA inactivation and semi-quinone formation, the hydrogen peroxide scavenger catalase was used. Catalase prevents formation of hydroxyl radicals by the Haber-Weiss reaction. As in the case of the addition of t-butanol, addition of 4000 U/ml catalase to the incubations of the ortho-quinone with ss ΦX174 DNA at pH 7.4 and pH 9 had no inhibitory effect on DNA inactivation. Also, no effects were observed by ESR and HPLC analysis on the respective semi-quinone and ortho-quinone signals. The effect of catalase on the DMPO–OH signals present in incubation of the ortho-quinone with DMPO at pH 7.4 and pH 9 was also investigated. 4000 U/ml catalase had no effect on the DMPO–OH signal present in incubation mixtures of the ortho-quinone after 10 min at pH 9 and after 1 hour at pH 7.4.

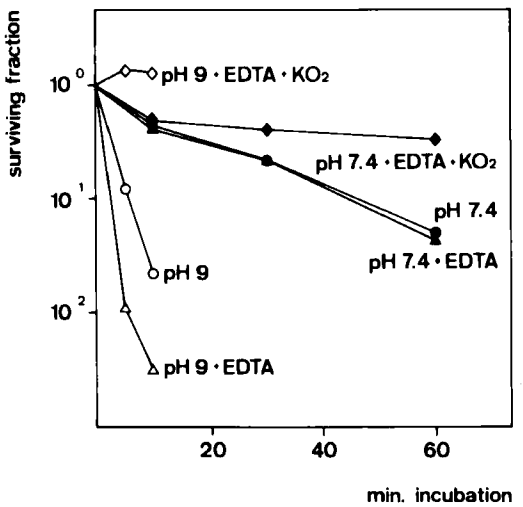

FIGURE 10 Survival curves of ss ΦX174 DNA incubated with the ortho-quinone of etoposide at pH 7.4, alone (●) and in the presence of 25 mM EDTA (▲) and 25 mM EDTA + 5 mM KO_2 (◆); and at pH 9, alone (○) and in the presence of 25 mM EDTA (△) and 25 mM EDTA and 5 mM KO_2 (◇).

KO_2

To examine the effects of $O_2^{\cdot}$ and $HO_2^{\cdot}$ on the DNA inactivation, KO_2 was used. This compound generates $O_2^{-\cdot}$ or $HO_2^{\cdot}$ in dependence of the pH used. KO_2 alone caused an extensive inactivation of DNA. When 25 mM EDTA was included in the reaction mixtures, in order to prevent formation of OH· radicals from KO_2 by the iron-catalyzed Haber-Weiss reaction, no DNA inactivation was observed anymore (results not shown). Further experiments were carried out in 5×10^{-2} M potassium phosphate buffers containing 25 mM EDTA. Figure 10 shows the survival curves of ss ΦX174 DNA incubated at pH 7.4 and at pH 9 with the ortho-quinone alone and in the presence of 25 mM EDTA plus 5 mM KO_2. EDTA did not protect the DNA from inactivation at pH 7.4 and pH 9. When 5 mM KO_2 was included in the incubation mixtures, the inactivation of DNA was inhibited. Interestingly, KO_2 had an increasing effect on DNA inactivation by the ortho-quinone at pH values < 7. As shown in Figure 11, in incubations at pH 6.5 the inactivation of DNA by the ortho-quinone was enhanced by the presence of 5 mM KO_2. At pH 4 the same increasing effect on DNA inactivation by KO_2 was observed, while no effect was found of KO_2 alone (data not shown).

ESR analysis showed that 25 mM EDTA had no effect on the intensity of the ESR signal of the semi-quinone radical generated from the ortho-quinone at pH 7.4 after 5 min of incubation. Addition of 5 mM KO_2 to the incubation of ortho-quinone at pH 7.4 had no effect on the semi-quinone signal present at $t = 0$, but after 5 min incubation at 37°C, the signal had disappeared, while it was still present in the incubation without KO_2. Addition of 0.5–1 mM KO_2 to a solution of the ortho-quinone in phosphate buffer pH 6.5 containing 25 mM EDTA, did not result in detection of a semi-quinone free radical signal.

The HPLC analysis showed that in incubations of the ortho-quinone at pH 6.5, 7.4 and 9 – which demonstrated an increased rate of disappearance of the ortho-quinone

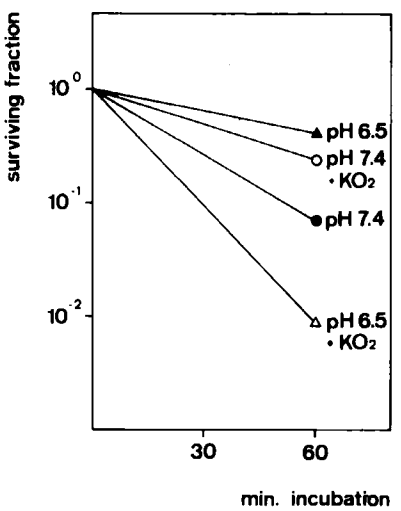

FIGURE 11 Survival curves of ss ΦX174 DNA incubated with the ortho-quinone of etoposide in pH 7.4/25 mM EDTA buffer, alone (●) and in the presence of 5 mM KO_2 (○); and in pH 6.5/25 mM EDTA buffer, alone (▲) and in the presence of 5 mM KO_2 (△).

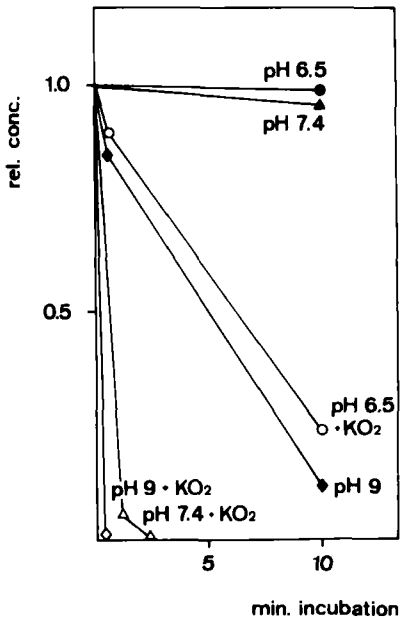

FIGURE 12 Decay of the ortho-quinone of etoposide during incubation at pH 6.5/25 mM EDTA, alone (●) and in the presecne of 5 mM KO_2 (○); at pH 7.4/25 mM EDTA, alone (▲) and in the presence of 5 mM KO_2 (△); and at pH 9/25 mM EDTA, alone (◆) and in the presence of 5 mM KO_2 (◇).

at increasing pH – in the presence of 5 mM KO_2, the rate of disappearance of the ortho-quinone at all pH values was considerably higher (Figure 12). This coincided with an increased rate of catechol formation in the presence of 5 mM KO_2 at decreasing pH (Figure 13).

In all of the described experiments, there was no DNA inactivation observed in control incubations of radical scavenger, spin trap or enzyme in the absence of ortho-quinone, except for the incubations with KO_2 in absence of EDTA.

DISCUSSION

We have previously observed that the catechol and ortho-quinone metabolites of etoposide – in contrast to the parent compound etoposide – cause inactivation of ss ΦX174 DNA.[14,16] Recently we reported that the inactivation of ss DNA by the ortho-quinone of etoposide increases to a considerable extent upon formation of the semi-quinone free radical from the ortho-quinone.[20] In the latter study it was reported that the rate of inactivation of ss ΦX174 DNA by the ortho-quinone of etoposide is higher at pH 9 than at pH 7.4. This has been explained to be due to increased formation of the semi-quinone free radical at pH 9, and the present study gives support for this explanation. In a recent extensive ESR study by Kalyanaraman *et al.*,[19] it was suggested that neither primary nor secondary radicals of etoposide react with DNA at a detectable rate. While this was clearly proven for the (primary and secondary) phenoxyl radicals of etoposide and is in line with our previous observations,[16] the arguments for lack of interaction of the semi-quinone free radicals with

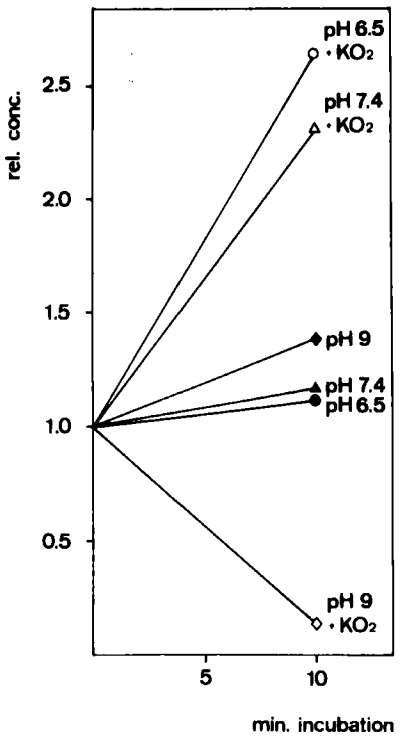

FIGURE 13 Formation of the catechol of etoposide during incubation of the ortho-quinone at pH 6.5/25 mM EDTA, alone (●) and in the presence of 5 mM KO_2 (○); at pH 7.4/25 mM EDTA, alone (▲) and in the presence of 5 mM KO_2 (△); and at pH 9/25 mM EDTA, alone (◆) and in the presence of 5 mM KO_2 (◇).

DNA were less clear. In the presence of DNA a decrease in the rate of production of the semi-quinone from the catechol was observed, which was explained by binding to DNA of the ortho-quinone formed from the semi-quinone. We observed stronger binding of the ortho-quinone to both single-stranded and double-stranded calf thymus DNA at higher pH values, where higher levels of the semi-quinone free radical are produced.[20,26] Recent results from our laboratory indicate that the (primary) semi-quinone free radical of etoposide does not inactivate double-stranded ΦX174 DNA.[26] Thus, the conclusions of Kalyanaraman *et al.* concerning the lack of reactivity of the semi-quinone free radical towards DNA might hold true for double-stranded, but not for single-stranded DNA. It should also be mentioned that inactivation of ΦX174 DNA is a more sensitive model to study interaction with DNA than binding to calf thymus DNA.

In the present study we investigated further the role of the semi-quinone free radical in the inactivation of ss ΦX174 DNA and also a possible contribution of oxygen radicals to DNA inactivation. These reactive oxygen species may be generated by redox cycling or by other chemical reactions involved in the formation of the semi-quinone free radical.[17] It has been well documented that oxygen radicals – in particular hydroxyl radicals – can play a role in DNA inactivation and cytotoxicity of quinoid anticancer agents.[21] Hydroxyl radicals could be formed in our system by

formation of $O_2^{-\cdot}$, followed by its dismutation to H_2O_2 and by the Haber-Weiss and Fenton reactions, leading to the formation of OH· radicals.[27] We used several hydroxyl radical scavengers and spin traps to study the possible role of hydroxyl radicals in the inactivation of DNA by the semi-quinone free radical: DMSO,[24] DMPO[28] and t-butanol;[25] we also used catalase and EDTA, which would inhibit hydroxyl radical formation by the iron-catalyzed Haber-Weiss reaction.

The hydroxyl radical scavenger t-butanol had no decreasing effect on DNA inactivation by the semi-quinone free radical, suggesting clearly that hydroxyl radicals are not involved in the process of DNA inactivation. Addition of catalase also had no decreasing effect on DNA inactivation by the semi-quinone free radical, suggesting that hydrogen peroxide – and indirectly hydroxyl radicals – are not involved in this process. Further support for this assumption was provided by the observation that addition of EDTA, which inhibits the metal-catalyzed formation of hydroxyl radicals from hydrogen peroxide, gave no protection of inactivation of DNA. Moreover, the hydroxyl radical scavenger DMSO did not inhibit DNA inactivation by the semi-quinone free radical, but on the contrary increased DNA inactivation, also arguing against the involvement of hydroxyl radicals. The increased DNA inactivation in the presence of DMSO could possibly be explained by the higher intensity of the semi-quinone free radical signal. An alternative explanation for the increased DNA inactivation in the presence of DMSO might be the formation of alkylperoxyl radicals due to reaction of OH· radicals with DMSO. However, this is unlikely in view of our results showing the lack of formation of hydroxyl radicals. The change in hyperfine splitting of the semi-quinone free radical signal might be a solvent effect of DMSO.[29] The faster decay of the ortho-quinone in the incubations with increasing concentrations DMSO could possibly be explained by a faster conversion to the semi-quinone radical in the presence of DMSO.

The spin trapping agent DMPO caused an extensive decrease in the DNA inactivation by the semi-quinone free radical and a decrease in the semi-quinone free radical signal. This was accompanied by the formation of a DMPO–OH adduct. Since the results from the DNA inactivation experiments excluded involvement of OH· radicals, this spin adduct probably does not arise from trapping of these radicals. Furthermore, addition of catalase – which inhibits the formation of hydroxyl radicals by the Fenton reaction by scavenging hydrogen peroxide – did not result in alteration of the DMPO–OH signal generated both at pH 7.4 and at pH 9. Also, addition of EDTA – which prevents hydroxyl radical formation – had no effect on the DMPO–OH signal. Alternative to the direct trapping of hydroxyl radicals, the formation of the DMPO–OH signal could be due to trapping of superoxide to DMPO–OOH, which decomposes rapidly to DMPO–OH.[30] It is known that hydroxyl radicals react with ethanol to produce α-hydroxyethyl radicals, which also react with the spin trap DMPO to produce an adduct with an ESR spectrum distinguishable from that of a hydroxyl radical adduct. Since addition of ethanol to the incubation mixture of ortho-quinone and DMPO also had no effect on the DMPO–OH signal, the possibility that the DMPO-adduct is formed directly by OH· can be ruled out. The signal, therefore, probably arises from trapping of $O_2^{-\cdot}$. In the recent study of Kalyanaraman *et al.*,[19] evidence was provided for the generation of hydroxyl radicals during autoxidation of the catechol of etoposide at alkaline pH. It should be mentioned that the system used differs from our system in the use of the substrate: catechol vs. ortho-quinone of etoposide. The difference in conclusion about hydroxyl radical production can probably be explained by the fact that the addition of iron chelates

was found to be a prerequisite for hydroxyl radical formation while in our experiments no iron chelates were used. The inhibitory effects of DMPO on DNA inactivation and semi-quinone formation which we observed were very strong, since the increasing effects of DMSO on DNA inactivation and semi-quinone formation could be blocked by DMPO. The three different peaks b present besides the DMPO–OH adduct in the ESR spectra of the incubations of the ortho-quinone at pH 7.4 and pH 9 (Figure 7) could be due to formation of another spin adduct. From these results the tentative conclusion could be drawn that the inhibition of DNA inactivation by DMPO was caused by the trapping of superoxide anion radicals, which could be involved in the formation of the semi-quinone free radical from the ortho-quinone. Superoxide dismutase had only a small inhibitory effect on DNA inactivation and semi-quinone formation at pH 9. However, previous studies showed that the ortho-quinone binds at a higher rate to proteins than to DNA: in 1 hour 40% of ortho-quinone was found to bind covalently to rat liver microsomal proteins vs. 0.5% to calf thymus DNA.[14] We therefore think that the effects of the inert molecules t-butanol, DMSO, DMPO and EDTA are more reliable than the effect of SOD, which could involve binding of the ortho-quinone to SOD.

To investigate further the possible role of superoxide anion radicals in the inactivation of DNA by the semi-quinone free radical, the effect of KO_2 on the inactivation was examined. KO_2 alone caused extensive inactivation of DNA, which was inhibited by EDTA. This inactivation by KO_2 must be due to the formation of hydroxyl radicals by the metal-catalyzed Haber-Weiss reaction.[31] It is clear that $O_2^{-\cdot}$ and $HO_2^{\cdot}$ themselves give no inactivation of DNA, but only after generation of $OH\cdot$ radicals. The fact that EDTA protected the $OH\cdot$ radical mediated DNA inactivation by KO_2 but not the inactivation by the semi-quinone free radical of etoposide, gives further support for the conclusion that hydroxyl radicals are not involved in the DNA inactivation by the semi-quinone free radical of etoposide. The addition of KO_2 to the incubation mixture of the ortho-quinone in the presence of DNA had an inhibitory effect on DNA inactivation at pH values $\geqslant 7.4$, but an increasing effect at pH values < 7.4. The HPLC and ESR analyses showed a faster disappearance of the ortho-quinone and semi-quinone at alkaline pH-values in the presence of KO_2. This might be due to a high reaction rate of the ortho-quinone with an excess of KO_2 at alkaline pH or a shorter life-time of the semi-quinone radical in the presence of an excess KO_2. On the contrary, in incubations of the ortho-quinone with KO_2 at pH value < 7.4, which showed an increased rate of DNA inactivation, a lower rate of disappearance of the ortho-quinone was observed than in the incubations at alkaline pH-values. This was accompanied by formation of the catechol of etoposide. The rate of inactivation of DNA by the catechol of etoposide is much lower than the rate of inactivation observed in the incubations of the ortho-quinone with KO_2 at pH < 7.4. Therefore, it could be suggested that the increase of DNA inactivation by the ortho-quinone at pH < 7.4 in the presence of KO_2 is due to formation of the semi-quinone free radical, although we were not able to observe a free radical signal in the incubation of the ortho-quinone at pH 6.5 in the presence of KO_2. However, the extensive formation of the catechol at pH 6.5 suggests that the semi-quinone free radical is reduced to the catechol by KO_2.

As we have previously reported, in the scheme of reactions of the ortho-quinone at alkaline pH – which lead to the formation of the semi-quinone free radical and secondary polar products – in the presence of oxygen probably the formation of superoxide anion radicals takes place.[17] The superoxide anion radicals could play a

role in the formation of the semi-quinone free radical by reduction of the ortho-quinone. It is known that superoxide anion radical can act as a reducing agent (e.g. the Haber-Weiss reaction or reduction of cytochrome c). The effects of DMPO could possibly be explained by trapping of superoxide, leading to a decreased semi-quinone formation. The effects of KO_2 might be explained by reduction of the ortho-quinone to the semi-quinone radical and to the catechol. It can be proposed that at alkaline pH values the presence of the excess superoxide anion radical causes the reactions to proceed fast with further conversion of the catechol; at lower pH values (pH 4–7), the less reactive $HO_2^{\cdot}$[31] gives slower reactions with catechol accumulation. This could explain the increased DNA inactivation, which is probably caused by the semi-quinone free radical ($HO_2^{\cdot}$ itself causes no DNA inactivation). In the recent study by Kalyanaraman *et al.*, it was suggested that superoxide is not involved in the formation of the (primary) semi-quinone free radical during autoxidation of the catechol. However, our experiments were performed with the ortho-quinone of etoposide and according to our data superoxide can play a role in the formation of the semi-quinone during (hydrolytic) oxidation of the ortho-quinone.

The lack of formation of hydroxyl radicals in this system could be due to the very high reactivity of the ortho-quinone with superoxide. Other investigators have reported that hydroxyl radical scavengers do not affect the DNA damaging effect of etoposide in L1210 cells as measured by the alkaline elution method,[32] and the results of the present study are in line with this observation. Our recent studies on the mechanism of inactivation of ss ΦX174 DNA by the ortho-quinone of etoposide at alkaline pH by sucrose gradient centrifugation of the DNA showed that no or a negligible amount of DNA breakage had occurred, which argues against the involvement of hydroxyl radicals.[20] In conclusion, the present study shows that the semi-quinone free radical of etoposide generated from the ortho-quinone plays an important role in ss DNA inactivation without involvement of hydroxyl radicals. Superoxide anions could be involved in the conversion of the ortho-quinone into the semi-quinone free radical, thus indirectly influencing DNA inactivation.

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