Biochemical Pharmacology, Vol. 43, No. 8, pp. 1761–1768, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

REACTIONS OF GLUTATHIONE WITH THE CATECHOL, THE *ortho*-QUINONE AND THE SEMI-QUINONE FREE RADICAL OF ETOPOSIDE

CONSEQUENCES FOR DNA INACTIVATION

D. R. A. MANS,* M. V. M. LAFLEUR, E. J. WESTMIJZE, I. R. HORN, D. BETS, G. J. SCHUURHUIS, J. LANKELMA and J. RETÈL
Department of Oncology, Free University Hospital, Amsterdam, The Netherlands

(*Received* 1 *August* 1991; *accepted* 9 *January* 1992)

Abstract—Etoposide [4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside)] can be metabolized to DNA-inactivating catechol, *ortho*-quinone and semi-quinone free radical derivatives which may contribute to its cytotoxicity. In this paper, we examined *in vitro* whether glutathione (GSH), which is known to react easily with quinoid compounds, could interact with the active etoposide intermediates and in this way influence the cytotoxicity of the parent compound. To this end, reactions of GSH with the etoposide intermediates were studied, using HPLC and ESR measurements, together with the effects of GSH on the biological inactivation of single-stranded (ss) and double-stranded (RF) ΦX174 DNA by these compounds. From the results it could be determined that: (a) GSH does not react with the catechol and, as a consequence, has no effect on the reaction of this intermediate of etoposide with ss and RF ΦX174 DNA; (b) GSH reacts with the *ortho*-quinone most likely by formation of a conjugate and by two-electron reduction to the catechol, resulting in a partial protection of ss and RF ΦX174 DNA against inactivation by this species; and (c) GSH protects ss ΦX174 DNA against inactivation by the semi-quinone free radical of etoposide probably by conjugation with this species.

Etoposide [4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside);† VP-16-213; NSC 141540] is used in the treatment of several malignant tumors, either as a single agent or in combination therapy [1]. The mechanism of action of this agent is generally believed to be based on the introduction of DNA damage [2–4]. In addition to interference by etoposide with the DNA breakage–reunion reaction of DNA topoisomerase II [5–7], this process could involve metabolic conversion of the drug into DNA-damaging intermediates. In cell-free systems, etoposide has been demonstrated to be metabolized by cytochrome P450 [8–12] and by peroxidases like prostaglandin E synthetase and myeloperoxidase [13–16] to a 3′,4′-dihydroxy derivative or catechol [9, 10], an *ortho*-quinone [17], a semi-quinone free radical [18], and a phenoxy radical [13]. With the exception of the latter species and of etoposide itself, all these intermediates of etoposide have been shown to inactivate biologically active ΦX174 DNA [9, 16–21] primarily by the formation of chemical DNA adducts [9–11, 14, 21].

Glutathione (GSH), which is present in all mammalian cells in relatively high concentrations, represents one of the defence mechanisms of cells against injury by various anticancer drugs, carcinogens, ionizing radiation and oxygen-derived free radical species [22–24]. This capacity of GSH is to a large degree due to its nucleophilic character, which enables it either to form conjugates with various xenobiotics and/or their metabolic intermediates, to scavenge possibly formed radical species or to convert these compounds into less toxic reduction products [22–24]. Moreover, the thiol is involved in chemical repair of damaged cellular constituents [22–24]. Since GSH is known to react easily with quinoid compounds, in particular [25–27], one might expect an interaction of this compound with the electrophilic intermediates of etoposide. The phenoxy radical has been demonstrated to react *in vitro* with GSH under the regeneration of the parent compound and the formation of oxidized glutathione (GSSG) [28]. This finding is of interest, since the phenoxy radical could serve as a precursor for the formation of the *ortho*-quinone and the semi-quinone free radical of etoposide [13]. Based on their strong electrophilic character, however, the latter intermediates of etoposide might also react with GSH. Such a process could have considerable implications for the antitumor activity of etoposide. We therefore examined a possible interaction of the *ortho*-quinone, the catechol and the semi-quinone free radical of etoposide with GSH using HPLC and ESR techniques. Furthermore, the effects of GSH on the inactivation of ΦX174 DNA caused by the three compounds was studied.

* Corresponding author: Dr D. R. A. Mans, Department of Oncology, BR 232, Free University Hospital, P.O.B. 7057, 1007 MB Amsterdam, The Netherlands.

† Abbreviations: etoposide, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside; GSH, glutathione; GSSG, oxidized glutathione; RF, replicative form; ss, single-stranded.

MATERIALS AND METHODS

Drugs and chemicals. Non-labeled etoposide was

generously supplied by the Bristol-Myers Co. (Syracuse, NY, U.S.A.). ^{3}H-Labeled etoposide (sp. act. 1 Ci/mmol) was from Moravek Biochemicals (Brea, CA, U.S.A.). (^{3}H-Labeled) *ortho*-quinone and catechol of etoposide were prepared as described before [29]. The formation and the purity of these species were checked by HPLC. Non-labeled GSH was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). ^{3}H-Labelled GSH (sp. act. 1 Ci/mmol) was from Du Pont de Nemours Nederland BV ('s Hertogenbosch, The Netherlands). All other chemicals used were reagent grade. Only freshly prepared (^{3}H-labeled) catechol or *ortho*-quinone of etoposide was used for the experiments.

Incubations were performed in 5×10^{-2} M potassium phosphate of which the pH values were adjusted with HCl or NaOH when necessary. To assure that no pH changes had taken place during the incubations, the pH values were checked before and after the incubations.

Incubations of the catechol, the ortho*-quinone and the semi-quinone free radical of etoposide with GSH.* A possible reaction of the catechol, the *ortho*-quinone and the semi-quinone free radical of etoposide with GSH was examined by incubating 5×10^{-4} M ^{3}H-labeled catechol or ^{3}H-labeled *ortho*-quinone for 15 min at 37° in the absence or presence of 5×10^{-4} M non-labeled GSH in 5×10^{-2} M potassium phosphate pH 4, 7.4 or 9 in a total volume of 150 μL. Of each of these reaction mixtures, 100 μL was analysed by HPLC (see below). To identify with more accuracy the possible reaction products of GSH with ^{3}H-labeled catechol or with ^{3}H-labeled *ortho*-quinone of etoposide, the above incubations were repeated with the non-labeled derivatives of etoposide, on the one hand, and ^{3}H-labeled GSH, on the other hand.

Additionally, a possible interaction of GSH with the semi-quinone free radical of etoposide was studied by ESR analyses (see below) of solutions of 5×10^{-4} M non-labeled catechol or *ortho*-quinone which were incubated for 15 min at 37° in the absence or presence of equimolar amounts of non-labeled GSH in 1 mL 5×10^{-2} M potassium phosphate pH 7.4 or 9.

Incubations with ΦX174 DNA. Biologically active single stranded (ss) and double-stranded, replicative form (RF) ΦX174 DNA were isolated from wild-type ΦX174 DNA bacteriophage according to Blok *et al.* [30] and Baas *et al.* [31], respectively. To investigate a possible influence of GSH on DNA inactivation by the catechol, the *ortho*-quinone and the semi-quinone free radical of etoposide, 12.5 ng ss or RF ΦX174 DNA was incubated with 4.4×10^{-4} M or 1.8×10^{-3} M catechol or *ortho*-quinone, respectively, in the absence or presence of equimolar concentrations of GSH at 37° in 1 mL 5×10^{-2} M potassium phosphate pH 4, 7.4 or 9. At different time intervals during the incubations, 20-μL DNA samples were taken to examine the time-course of DNA inactivation. Reactions were terminated by immediate chilling on ice and 50-fold dilution with 2.5×10^{-2} M ice-cold Tris–HCl pH 8.3.

HPLC analyses. HPLC analyses were performed with a C18 Microsphere column (3 μm, 100 × 4.6 mm; Chrompack, Middelburg, The Netherlands) connected to a Perkin–Elmer Series 2 pump (Perkin–Elmer, Norwalk, CO, U.S.A.). The flow rate was 0.5 mL/min and the mobile phase consisted of 40% (*v*/*v*) methanol containing 5×10^{-2} M potassium phosphate pH 4. Fractions of 0.5 min were collected, in which the radioactivity was determined by addition of 5 mL Opti-Phase "Hi-Safe 3" (LKB, Bromma, Sweden) and counting in a Wallac Rackbeta Liquid Scintillation Counter (LKB, Bromma, Sweden). The radioactivity in each fraction was expressed as a percentage of the total amount of radioactivity which was injected on the column. Chromatographs were obtained by plotting these data versus elution time.

ESR measurements. ESR measurements were performed with an ESP-300 Spectrometer (Bruker, Rheinstetten, Germany). ESR spectra were recorded at room temperature in an ER 4102 standard rectangular cavity. Spectral intensities were calculated with an ESP 1600 Data Processing System (Bruker, Rheinstetten, Germany), utilizing double integration of the first derivative signal. Instrumental conditions: magnetic field: 3460 G; scan range: 20 G; modulation frequency: 100 kHz; modulation amplitude: 0.197 G; gain: 3.20×10^5; power: 50 mW; conversion time: 10.24 msec; number of scans: 20; scan time: 40 sec.

Determination of the biological activity of ΦX174 DNA. The rates of DNA inactivation were determined by transfection of the DNA samples to *Escherichia coli* spheroplasts as described previously [17] and by plotting semilogarithmically the surviving fraction of DNA versus incubation time. In the case of the incubations of ss ΦX174 DNA at pH 4 only, the survival curves had to be corrected for background effects due to the introduction of apurinic sites by the acidic incubation alone (correction factors up to 40%).

RESULTS

Reaction kinetics of GSH with the catechol, the ortho*-quinone and the semi-quinone free radical of etoposide*

The reactions with the catechol and the *ortho*-quinone of etoposide were studied at pH 4 and those with the semi-quinone free radical at pH 7.4 and 9, since previous results demonstrated that the former two species are chemically stable at acidic pH and generate the radical at pH values ≥7.4 [18, 20, 32]. The optimum conditions for examination of the reactions between GSH and the etoposide intermediates were determined by HPLC and ESR analysis. At pH 4, neither the catechol nor the *ortho*-quinone generated an ESR signal. When the catechol was incubated at pH 4 for 1 hr with a 10-fold excess of GSH, no alterations in its peak were found upon HPLC analysis. In contrast, the HPLC peak of the *ortho*-quinone at pH 4, as well as the ESR spectrum of the semi-quinone free radical generated from either the catechol or the *ortho*-quinone at pH 9, disappeared beyond the detection limit after incubation for 10 min in the presence of equimolar concentrations of GSH. To avoid an excess of the etoposide intermediates during incubation with ΦX174 DNA in the presence of GSH, we decided from these findings to examine the interaction of these compounds with GSH after incubation for

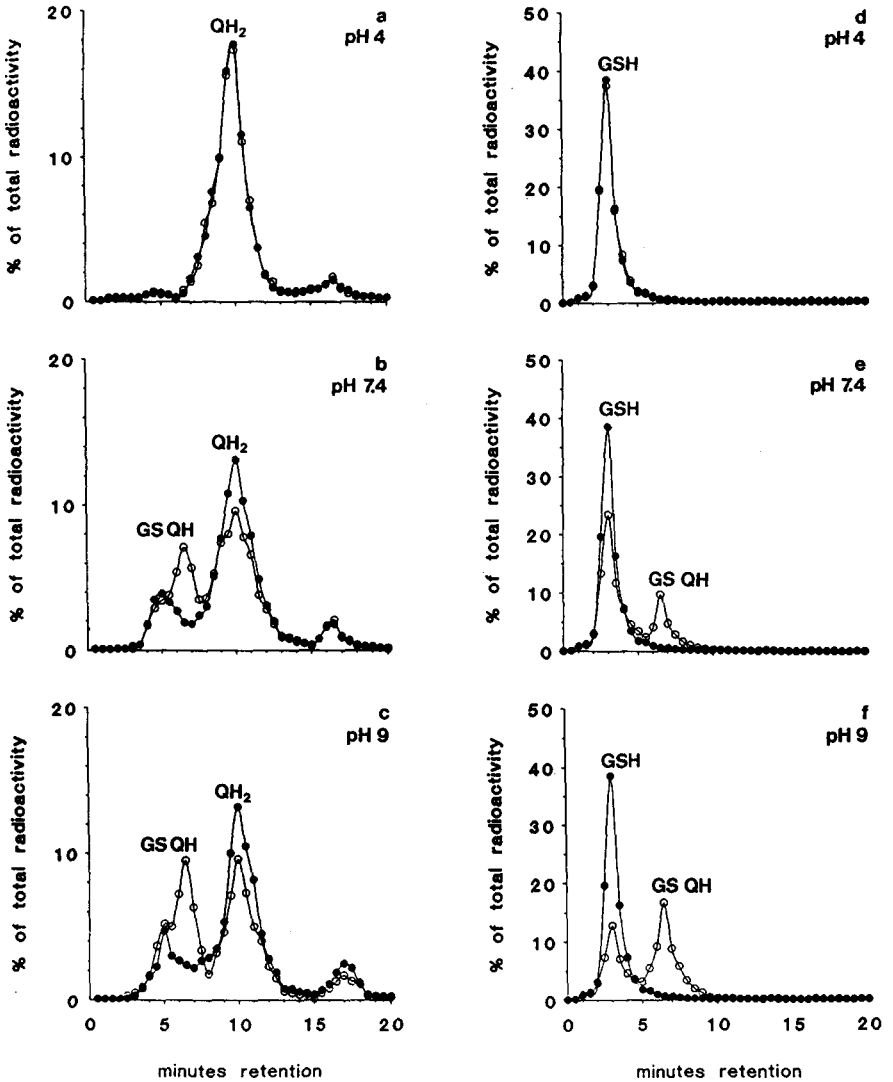

Fig. 1. HPLC analyses of ^{3}H-labeled catechol of etoposide (5×10^{-4} M) after incubation for 15 min at 37° without (closed symbols) or with equimolar concentrations of non-labeled GSH (open symbols) in 5×10^{-2} M potassium phosphate pH 4 (a), 7.4 (b) or 9 (c) and of non-labeled catechol of etoposide incubated under the same conditions with ^{3}H-labeled GSH at pH 4 (d), 7.4 (e) or 9 (f). GS QH, GSH conjugate; QH_2, catechol. The identity of the ^{3}H-labeled compounds with retention times ≤ 5 min and 16.5 min is mentioned in Results.

15 min with equimolar concentrations of each reacting species.

Reaction products of GSH with the ortho*-quinone, the catechol and the semi-quinone free radical of etoposide*

To investigate which products are formed after reaction of the *ortho*-quinone, the catechol and the semi-quinone free radical of etoposide with GSH, the ^{3}H-labeled etoposide derivatives were incubated with non-labeled GSH and *vice versa*, followed by HPLC analyses. In addition, ESR measurements were performed to examine the effects of GSH on the semi-quinone free radical.

No alterations were observed in the HPLC peak of either ^{3}H-labeled catechol of etoposide (Fig. 1a; retention time of 10 min) incubated with non-labeled GSH, or ^{3}H-labeled GSH (Fig. 1d; retention time of 3 min) incubated with non-labeled catechol at pH 4. Apparently, GSH does not react with the catechol under these conditions.

In contrast, incubation of ^{3}H-labeled *ortho*-quinone with non-labeled GSH and *vice versa* at pH 4 led to disappearance of the *ortho*-quinone (Fig. 2a; retention time of 8 min), a decrease in the amount of GSH (Fig. 2d; retention time of 3 min) and formation of a compound with a retention time of 6.5 min (Fig. 2a and d). Since this compound contained radioactivity from ^{3}H-labeled *ortho*-quinone as well as from ^{3}H-labeled GSH, and since its retention time was intermediate between that of the two reacting species, a conjugation product of

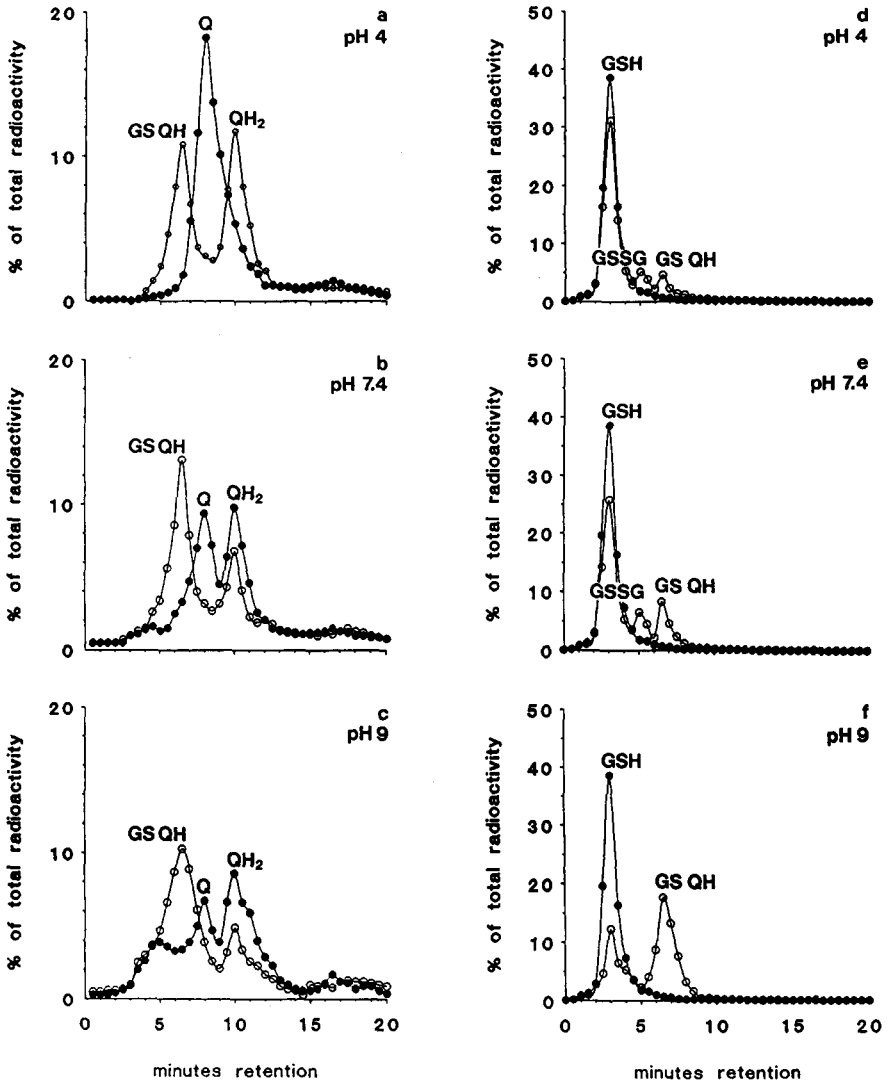

Fig. 2. HPLC analyses of ^{3}H-labeled *ortho*-quinone of etoposide (5×10^{-4} M) after incubation for 15 min at 37° without (closed symbols) or with equimolar concentrations of non-labeled GSH (open symbols) in 5×10^{-2} M potassium phosphate pH 4 (a), 7.4 (b) or 9 (c) and of non-labeled *ortho*-quinone of etoposide incubated under the same conditions with ^{3}H-labeled GSH at pH 4 (d), 7.4 (e) or 9 (f). GS QH, GSH conjugate; Q, *ortho*-quinone; QH_2, catechol. The identity of the ^{3}H-labeled compounds with retention times ≤ 5 and 16.5 min is mentioned in Results.

GSH with the *ortho*-quinone is strongly suggested. In addition, a compound which co-eluted with the catechol of etoposide (Fig. 2a) and a compound which co-eluted with GSSG (Fig. 2d; retention time of 5 min) were produced, showing that GSH also reduces the *ortho*-quinone to the catechol.

ESR measurements of catechol or *ortho*-quinone solutions at pH 7.4 or 9 in the absence of GSH showed formation of an ESR signal (not shown) which represented the semi-quinone free radical of etoposide [20]. The signal was completely quenched in the presence of GSH (not shown). HPLC analyses of ^{3}H-labeled catechol incubations at pH 7.4 or 9 with non-labeled GSH or *vice versa* showed decreased amounts of catechol and GSH, as well as the apperance of more polar radioactively labeled compounds with a retention time of again 6.5 min (Fig. 1b, c, e and f). Since in the absence of GSH no *ortho*-quinone was formed from the catechol, it is strongly suggested, according to the same rationale as above, that the reaction of GSH with the semi-quinone free radical also involves conjugate formation. The lack of formation of GSSG suggests, in addition, that reduction of the semi-quinone free radical by GSH to the catechol does not take place.

Incubation of (^{3}H-labeled) *ortho*-quinone with (^{3}H-labeled) GSH at pH 7.4 or 9, followed by HPLC analyses, showed complete disappearance of the *ortho*-quinone and a decrease in the amount of GSH, and furthermore the appearance of a peak with the

characteristics of a semi-quinone free radical- and/or *ortho*-quinone–GSH conjugate (retention time of 6.5 min) and of the catechol of etoposide (Fig. 2b, c, e and f). The latter compound was also formed (in even greater amounts) in the absence of GSH (Fig. 2b and c). The decreased amounts of catechol observed in the presence of GSH (Fig. 1b and c) are probably due to acceleration of semi-quinone free radical generation from the catechol, caused by removal of the radical by GSH. As was the case at pH 4, GSSG was also formed at pH 7.4 (Fig. 2e), demonstrating that some of the catechol production was due to GSH-mediated reduction under these conditions. At pH 9, no GSSG formation was detected (Fig. 2f). This observation, together with the fact that at this pH value the semi-quinone free radical is the main electrophilic compound in the incubations [20], again strongly suggests that reduction of this radical species by GSH does not take place.

The ^{3}H-labeled compounds with retention times $\leq$ 5 min, observed upon HPLC analysis of pH 7.4- and pH 9-solutions of ^{3}H-labeled *ortho*-quinone and catechol (Figs 1a–f and 2a–f), represent more polar conversion products produced during generation of the semi-quinone free radical [18, 20]. The ^{3}H-labeled compounds with a retention time of 16.5 min (Figs 1a–f and 2a–f) represent ^{3}H-labeled etoposide which was not converted during the preparation of ^{3}H-labeled *ortho*-quinone and ^{3}H-labeled catechol.

Effects of GSH on the inactivation of ΦX174 DNA by the ortho*-quinone, the catechol and the semi-quinone free radical of etoposide*

To examine the effects of GSH on the DNA inactivation by the three reactive etoposide intermediates, ss and RF ΦX174 DNA were incubated with the *ortho*-quinone or catechol at pH 4, 7.4 and 9 either in the absence or presence of GSH.

Co-incubation with GSH did not affect the inactivation of ss and RF ΦX174 DNA by the catechol itself (pH 4; Fig. 3a). At pH 7.4 and 9, the biological activity of the DNA was partially protected in the presence of GSH; this effect was greater at pH 9 than at pH 7.4, in particular in the case of ss ΦX174 DNA (Fig. 3a and b).

The presence of GSH in the incubation of ss ΦX174 DNA with the *ortho*-quinone at pH 4 and 7.4 led to decreased DNA inactivation (Fig. 4a). At pH 9, the activation of ss ΦX174 DNA was completely abolished by GSH (Fig. 4a). RF ΦX174 DNA was partially protected by GSH from inactivation by the *ortho*-quinone of etoposide at pH 4 (Fig. 4b). At pH 7.4 and 9, the biological activity of the DNA was unaffected in the absence or presence of GSH (Fig. 4b).

The more polar conversion products formed from the *ortho*-quinone and the catechol at pH values $\geq$ 7.4 (see preceding paragraph), as well as etoposide itself, were shown previously to be without significant effect on the biological activity of ΦX174 DNA during the time-course of the measurements [19, 20].

DISCUSSION

GSH plays an important role in the maintenance of cellular integrity through detoxification of various toxic species, including several quinoid chemotherapeutic compounds and/or their metabolites [22–24]. As a consequence, cellular GSH can seriously influence the efficacy of anticancer treatment [22–24]. Studies with cell-free *in vitro* systems [9, 17, 19–21, 33, 34], with cultured tumor cells [35–37] and with tumor-bearing mice [36, 37] suggest that potential metabolites of etoposide like the catechol, the *ortho*-quinone and the semi-quinone free radical may contribute to the cytotoxicity of the drug. Administration of etoposide to mice was observed to decrease GSH and to increase GSSG levels in the liver [28]. Since GSH does not react with etoposide itself, this observation suggests that GSH is able to react with one or more metabolites of the drug. The results presented in this paper strengthen this suggestion, since they show that GSH, although not affecting the reaction of the catechol of etoposide with ΦX174 DNA, protects the DNA from biological inactivation by the *ortho*-quinone and the semi-quinone free radical derivatives of etoposide.

These findings can be explained by the nucleophilic character of GSH, which enables the reaction with electrophilic *ortho*-quinones and semi-quinone free radicals according to conjugation and reduction processes of the types presented in the scheme below [25–27]. The abbreviations used are: Q, ortho-quinone; $QH^{\cdot}$, semi-quinone free radical; QH_2, catechol; GS QH, GSH conjugate.

(a) Conjugation reactions with an *ortho*-quinone:

$$GS^- + H^+ + Q \rightarrow GS\,QH.$$

(b) Conjugation reactions with a semi-quinone free radical:

$$GS^- + H^+ + QH^{\cdot} \rightarrow GS\,QH + H^+ + e^-.$$

(c) One-electron reduction reactions with an *ortho*-quinone:

$$GS^- + GS^+ + 2\,H^+ + 1\,e^- + 2\,Q \rightarrow GSSG + 2\,QH^{\cdot}.$$

(d) Two-electron reduction reactions with an *ortho*-quinone:

$$GS^- + GS^+ + 2\,H^+ + 2\,e^- + 2\,Q \rightarrow GSSG + 2\,QH_2.$$

(e) One-electron reduction reactions with a semi-quinone free radical:

$$GS^- + GS^+ + 2\,H^+ + 2\,e^- + 2\,QH^{\cdot} \rightarrow GSSG + QH_2.$$

Reactions of GSH with the catechol of etoposide and with etoposide itself, on the other hand, are less likely due to the non-electrophilic character of these species [25–27]. In agreement with this, the results from the HPLC studies showed no effect of GSH on the catechol at pH 4 (Fig. 1a and d), while the catechol-induced inactivation of ss and RF ΦX174 DNA was not affected by GSH at this pH value (Fig. 3a and b).

At pH 7.4 or 9, the catechol generates the semi-quinone free radical [18, 32] which is eliminated in the presence of GSH, as observed by ESR. This process was accompanied by the formation of a

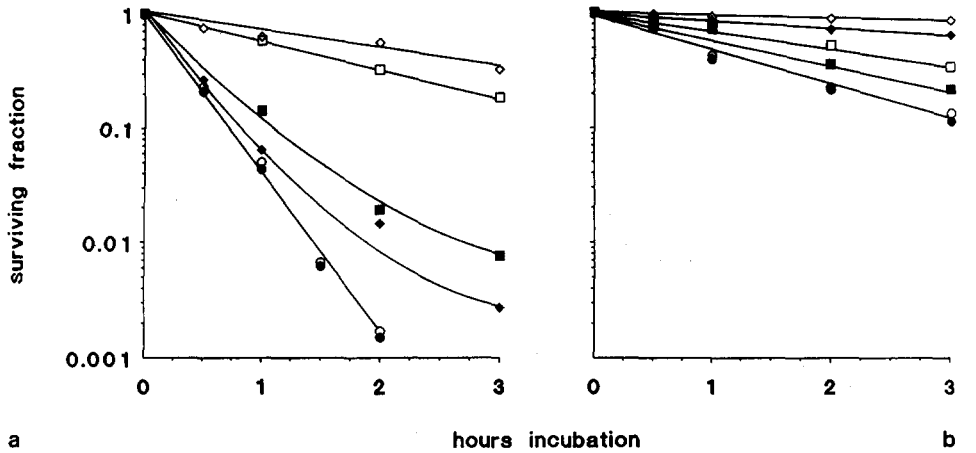

Fig. 3. Representative survival curves of ss (a) and RF (b) ΦX174 DNA (12.5 ng/mL) incubated at 37° with 4.4×10^{-4} M or 1.8×10^{-3} M catechol of etoposide, respectively, in 5×10^{-2} M potassium phosphate pH 4 (●—○), 7.4 (■—□) or 9 (◆—◇) in the absence (closed symbols) or in the presence of equimolar concentrations GSH (open symbols). The survival curves of RF ΦX174 DNA showed a variability of about 50%, probably due to variations in the purity of the catechol preparations. Nevertheless, in all experiments comparable effects on the biological activity of RF DNA were observed in the presence of GSH. The survival curves obtained upon incubation of ss ΦX174 DNA at pH 4 had to be corrected for background effects due to the introduction of apurinic sites by the acid incubation alone (correction factors of about 40%).

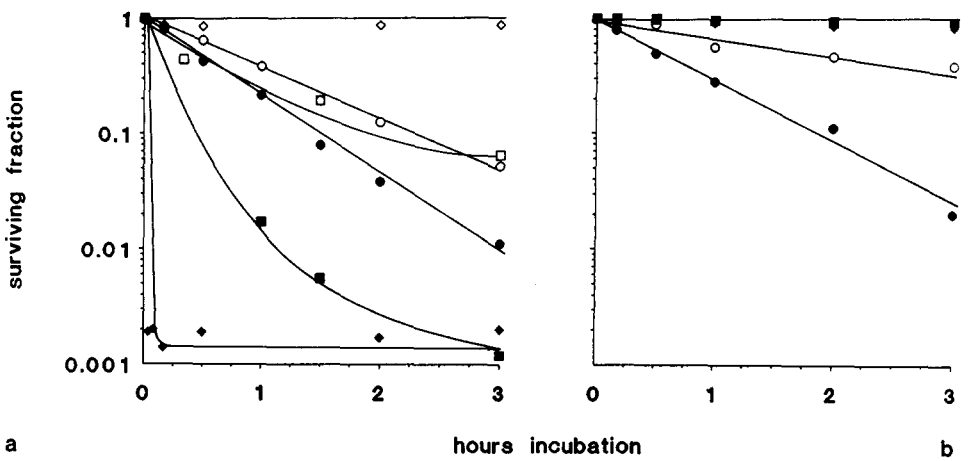

Fig. 4. Representative survival curves of ss (a) and RF (b) ΦX174 DNA (12.5 ng/mL) incubated at 37° with 4.4×10^{-4} M or 1.8×10^{-3} M *ortho*-quinone of etoposide, respectively, in 5×10^{-2} M potassium phosphate pH 4 (●—○), 7.4 (■—□) or 9 (◆—◇) in the absence (closed symbols) or in the presence of equimolar concentrations GSH (open symbols). The survival curves obtained upon incubation of ss ΦX174 DNA at pH 4 had to be corrected for background effects due to the introduction of apurinic sites by the acidic incubation alone (correction factors of about 40%).

compound which, according to its chromatographic properties and to the distribution of radioactivity, might well represent a conjugation product of GSH with the semi-quinone free radical (Fig. 1b, c, e and f). Such a species could be produced according to reaction (b). Removal of the semi-quinone free radical from the incubations by conjugation with GSH will stimulate semi-quinone free radical generation from the catechol, resulting in a further decrease in the amounts of catechol in the incubations (Fig. 1b and c). This can explain the partial protection of the biological activity of ss and RF ΦX174 DNA observed upon incubation with the catechol at pH 7.4 and 9 in the presence of GSH (Fig. 3a and b). The absence of GSSG in these incubations (Fig. 2e and f) strongly suggests that GSH does not react with the semi-quinone free radical via reduction according to reaction (e). The large degree of protection of

the biological activity of the DNA observed in the presence of GSH indicates, in addition, that the formed GSH conjugate does not contribute significantly to DNA inactivation.

In contrast to the catechol, the *ortho*-quinone is able to react with GSH. The results from the HPLC analyses showed that at pH 4 about 50% of the *ortho*-quinone was converted into a compound which may represent a conjugation product of the *ortho*-quinone with GSH (Fig. 2a), judged from the HPLC characteristics which were similar to those found for the GSH–semi-quinone free radical conjugate. Such a species could be formed according to reaction (a). The other half of the *ortho*-quinone was converted into the catechol (Fig. 1a) under the formation of GSSG (Fig. 1d). The GSSG production upon *ortho*-quinone-catalysed oxidation of GSH [reactions (c) and (d)] may have proceeded through formation of unstable GSH conjugates, formed in reactions of type (a). A more likely explanation is that GSH is also able to react with the *ortho*-quinone—in contrast to the semi-quinone free radical—via reduction, probably by two-electron transfer according to reaction (d), resulting in the formation of the catechol of etoposide. The catechol has a DNA-inactivating capacity comparable with that of the *ortho*-quinone (Fig. 3a and b) while the GSH conjugate does not inactivate DNA (as argued above). The rate and degree of DNA inactivation under such experimental conditions will thus be reduced by about one half. Our results indeed show protection of about 50% of the biological activity of ss and RF ΦX174 DNA incubated at pH 4 with the *ortho*-quinone in the presence of GSH (Fig. 4a and b). One-electron reduction of the *ortho*-quinone by GSH [reaction (c)] could not be established, due to the relatively fast elimination of the resulting semi-quinone free radical by GSH (not shown).

In the pH 7.4- and pH 9-incubations with the *ortho*-quinone and GSH, both the *ortho*-quinone (Fig. 2b, c, e and f) and the concomitantly generated semi-quinone free radical were eliminated by the thiol (not shown). DNA incubated under such conditions thus underwent inactivation by only relatively small amounts of catechol left behind in the incubations, especially in those at pH 9. This can account for the partial or almost complete protection of the biological activity of ss ΦX174 DNA incubated with the *ortho*-quinone at pH 7.4 or 9, respectively, in the presence of GSH (Fig. 4a). The lack of an effect of GSH on the biological activity of RF ΦX174 DNA under these conditions (Fig. 4b) is not surprising, since the DNA had not been significantly inactivated by the concentrations of semi-quinone free radical, *ortho*-quinone and catechol in these incubations in the absence of the thiol (Fig. 4b) [20, 21].

In summary, the data presented in this study show that: (a) GSH does not react with the catechol, hence its lack of effect on the reaction of this derivative of etoposide with ss and RF ΦX174 DNA; (b) GSH protects ss and RF ΦX174 DNA against inactivation by the *ortho*-quinone of etoposide, most likely via conjugation and two-electron reduction to the catechol derivative under the formation of GSSG; and (c) GSH protects ss ΦX174 DNA against inactivation by the semi-quinone free radical of etoposide via probably conjugation only.

Since several lines of evidence indicate that the cytotoxicity of etoposide could depend to a large degree on metabolic conversion into reactive intermediates, the reduction and conjugation processes described in the present *in vitro* study might well occur in cells as well as *in vivo*. It should be noted, however, that *in vivo* the largest component of GSH conjugation can be ascribed to enzymatic catalysis by GSH-*S*-transferases. This implies that in cells, apart from non-enzymatic conversion (as described in the present paper), enzyme-mediated conjugation of the *ortho*-quinone and the semi-quinone free radical of etoposide could take place, which may result in very different reaction products.

The results from the present study suggest that cellular GSH content may be a determinant for etoposide cytotoxicity. Since this finding could have important implications for clinical application of the drug, we initiated studies on the effect of altered GSH levels on the cytotoxicity of etoposide to cultured tumor cells. The results from those studies indeed showed an enhanced sensitivity to etoposide in cultured tumor cells which are depleted of GSH [38]. In cells, there will probably be a huge excess of GSH compared to the etoposide intermediates. Moreover, GSH could very well contain more than one conjugation site with the etoposide derivatives. This implies that mechanisms additional to a decreased elimination of potentially cytotoxic etoposide metabolites could contribute to the potentiation of etoposide cytotoxicity observed in GSH-depleted cells. A detailed investigation of this phenomenon will be published elsewhere [38].

Acknowledgement—This work was supported by the Netherlands Cancer Foundation (grant IKA 85-06).

REFERENCES

1. Issell BF, Muggia FM and Carter SK, *Etoposide (VP-16). Current Status and New Developments.* Academic Press, Orlanda, U.S.A., 1984.
2. Huang CC, Hou Y and Wang JJ, Effects of a new antitumor agent, epipodophyllotoxin, on growth and chromosomes in human hematopoietic cell lines. *Cancer Res* **33**: 3123–3129, 1973.
3. Loike JD and Horwitz SB, Effect of VP-16-213 on the intracellular degradation of DNA in HeLa cells. *Biochemistry* **15**: 5443–5448, 1976.
4. Wozniak AJ and Ross WE, DNA damage as a basis for 4′-demethyl-epipdophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside) (etoposide) cytotoxicity. *Cancer Res* **43**: 120–124, 1983.
5. Glisson BS, Smallwood SE and Ross WE, Characterization of VP-16-induced DNA damage in isolated nuclei from L1210 cells. *Biochim Biophys Acta* **783**: 74–79, 1984.
6. Ross WE, Rowe T, Glisson BS, Yalowich J and Liu L, Role of topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage. *Cancer Res* **44**: 5857–5860, 1984.
7. Chen GL, Yang L, Rowe TC, Halligan BD, Tewey KM and Liu LF, Non-intercalative antitumor drugs interfere with the breakage–reunion reaction of mammalian DNA topoisomerase II. *J Biol Chem* **259**: 13560–13566, 1984.
8. van Maanen JMS, de Ruiter C, de Vries J, Kootstra

PR, Gobas F and Pinedo HM, The role of metabolic activation by cytochrome P-450 in covalent binding of VP-16-213 to rat liver and HeLa cell microsomal proteins. *Eur J Cancer Clin Oncol* **21**: 1099–1106, 1985.
9. van Maanen JMS, de Vries J, Pappie D, Mans DRA, van den Akker E, Lafleur MVM, Retèl J, van der Greef J and Pinedo HM, Cytochrome P-450-mediated O-demethylation: a route in the metabolic activation of etoposide. *Cancer Res* **47**: 4658–4662, 1987.
10. Haim N, Nemec J, Roman J and Sinha BK, *In vitro* metabolism of etoposide (VP-16-213) by liver microsomes and irreversible binding of reactive intermediates to microsomal proteins. *Biochem Pharmacol* **36**: 527–536, 1987.
11. Sinha BK and Myers CE, Irreversible binding of etoposide (VP-16-213) to deoxyribonucleic acid and proteins. *Biochem Pharmacol* **33**: 3725–3728, 1984.
12. Sinha BK, Trush MA and Kalyanaraman B, Microsomal interactions and inhibition of lipid peroxidation by etoposide (VP-16-213): implications for mode of action. *Biochem Pharmacol* **34**: 2036–2040, 1985.
13. Haim N, Roman J, Nemec J and Sinha BK, Peroxidative free radical formation and O-demethylation of etoposide (VP-16) and teniposide (VM-26). *Biochem Biophys Res Commun* **135**: 215–220, 1986.
14. Haim N, Nemec J, Roman J and Sinha BK, Peroxidase-catalyzed metabolism of etoposide (VP-16-213) and covalent binding of reactive intermediates to cellular macromolecules. *Cancer Res* **47**: 5835–5840, 1987.
15. Sinha BK, Trush MA and Kalyanaraman B, Free radical metabolism of VP-16 and inhibition of anthracycline-induced lipid peroxidation. *Biochem Pharmacol* **32**: 3495–3498, 1983.
16. van Maanen JMS, de Ruiter C, Kootstra PR, de Vries J and Pinedo HM, Free radical formation from the antineoplastic agent VP-16-213. *Free Rad Res Commun* **1**: 263–272, 1986.
17. van Maanen JMS, Lafleur MVM, Mans DRA, van den Akker E, de Ruiter C, Kootstra PR, Pappie D, de Vries J, Retèl J and Pinedo HM, Effects of the *ortho*-quinone and catechol of the anti-tumor drug VP-16-213 on the biological activity of single-stranded and double-stranded ΦX174 DNA. *Biochem Pharmacol* **37**: 3579–3589, 1988.
18. van Maanen JMS, Verkerk UH, Broersen J, Lafleur MVM, de Vries J, Retèl J and Pinedo HM, Semi-quinone formation from the catechol and *ortho*-quinone metabolites of the antitumor agent VP-16-213. *Free Rad Res Commun* **6**: 371–384, 1988.
19. van Maanen JMS, de Ruiter C, Kootstra PR, Lafleur MVM, de Vries J, Retèl J and Pinedo HM, Inactivation of ΦX174 DNA by the *ortho*-quinone derivative or its reduction product of the antitumor agent VP-16-213. *Eur J Cancer Clin Oncol* **21**: 1215–1218, 1985.
20. Mans DRA, Retèl J, van Maanen JMS, Lafleur MVM, van Schaik MA, Pinedo HM and Lankelma J, Role of the semi-quinone free radical of the antitumor agent etoposide (VP-16-213) in the inactivation of single- and double-stranded ΦX174 DNA. *Br J Cancer* **62**: 54–60, 1990.
21. Mans DRA, Lafleur MVM, Westmijze EJ, van Maanen JMS, van Schaik MA, Lankelma J and Retèl J, Formation of different reaction products with single- and double-stranded DNA by the *ortho*-quinone and the semi-quinone free radical of etoposide (VP-16-213). *Biochem Pharmacol* **42**: 2131–2139, 1991.
22. Kosower NS and Kosower EM, The glutathione status of cells. *Int Rev Cytol* **54**: 109–160, 1978.
23. Meister A and Anderson ME, Glutathione. *Annu Rev Biochem* **52**: 711–760, 1983.
24. Orrenius S and Moldéus P, The multiple roles of glutathione in drug metabolism. *Trends Pharmacol Sci* **5**: 432–435, 1984.
25. Kosower EM, Chemical properties of glutathione. In: *Glutathione: Metabolism and Function* (Eds. Arias IM and Jakoby WB), pp. 1–15. Raven Press, New York, 1976.
26. Chasseuad LF, Conjugation with glutathione and mercapturic acid excretion. In: *Glutathione: Metabolism and Function* (Eds. Arias IM and Jakoby WB), pp. 78–114. Raven Press, New York, 1976.
27. Lunte SM and Kissinger PT, Detection and identification of sulfhydryl conjugates of *p*-benzoquinones in microsomal incubations of benzene and phenol. *Chem Biol Interact* **47**: 195–212, 1983.
28. Katki AG, Kalyanaraman B and Sinha BK, Interactions of the antitumor drug, etoposide, with reduced thiols *in vitro* and *in vivo*. *Chem Biol Interact* 62: 237–247, 1987.
29. Holthuis JJM, van Oort WJ, Römkens FMGM, Renema J and Zuman P, Electrochemistry of podophyllotoxin derivatives. I. Oxidation mechanism of etoposide (VP-16-213). *J Electroanal Chem* **184**: 317–329, 1985.
30. Blok J, Luthjens LH and Roos ALM, The radiosensitivity of bacteriophage DNA in aqueous solution. *Radiat Res* **30**: 468–482, 1967.
31. Baas PD, Teertstra WR, van Mansveld ADM and Jansz HS, Construction of viable and lethal mutations in the origin of bacteriophage ΦX174 using synthetic oligo-deoxyribonucleotides. *J Mol Biol* **152**: 615–639, 1981.
32. Kalyanaraman B, Nemec J and Sinha BK, Characterization of free radicals produced during oxidation of etoposide (VP-16) and its catechol and quinone derivatives. An ESR study. *Biochemistry* **28**: 4839–4846, 1989.
33. Sinha BK, Elliot HM and Kalyanaraman B, Iron-dependent hydroxyl radical formation and DNA damage from a novel metabolite of the clinically active antitumor drug VP-16. *FEBS Lett* **227**: 240–244, 1988.
34. Sinha BK, Politi PM, Eliot HM, Kerrigan D and Pommier Y, Structure–activity relations, cytotoxicity and topoisomerase II dependent cleavage induced by pendulum ring analogues of etoposide. *Eur J Cancer* **26**: 590–593, 1990.
35. Wozniak AJ, Glisson BS, Hande KR and Ross WE, Inhibition of etoposide-induced DNA damage and cytotoxicity in L1210 cells by dehydrogenase inhibitors and other agents. *Cancer Res* **44**: 626–632, 1984.
36. Teicher BA, Holden SA and Rose CM, Effect of oxygen on the cytotoxicity and antitumor activity of etoposide. *J Natl Cancer Inst* **75**: 1129–1133, 1985.
37. Teicher BA, Bernal SD, Holden SA and Cathcart KNS, Effect of fluosol-DA/carbogen on etoposide/alkylating agent antitumor activity. *Cancer Chemother Pharmacol* **21**: 281–285, 1988.
38. Mans DRA, Schuurhuis GJ, Treskes M, Lafleur MVM, Retèl J, Pinedo HM and Lankelma J, Modulation of etoposide cytotoxicity by D,L-buthionine-*S*,*R*-sulfoximine treatment in human non-small cell lung, ovarian and breast carcinoma cell lines. *Eur J Cancer*, in press.