BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **237,** 24–27 (1997)
ARTICLE NO. RC977063

Inhibition of the Topoisomerase II-DNA Cleavable Complex by the *ortho*-Quinone Derivative of the Antitumor Drug Etoposide (VP-16)

Tsvetan G. Gantchev[1] and Darel J. Hunting
MRC Group in the Radiation Sciences, Department of Nuclear Medicine and Radiobiology, Faculty of Medicine, University of Sherbrooke, Sherbrooke (PQ) J1H 5N4, Canada

Received June 17, 1997

Etoposide (VP-16) is a widely used anticancer drug whose toxicity involves poisoning of topoisomerase II. VP-16 undergoes enzymatic oxido-reductive transformations in cells, resulting in the formation of the *ortho*-quinone derivative (VPQ) as a major product. The actions of VP-16 and VPQ on purified human topoisomerase II have been compared. Both the parent drug and VPQ are very efficient at trapping the topoisomerase II-DNA cleavable complex, suggesting that methoxy groups on the E-ring are not a prerequisite for activity. Our data also imply that VPQ has more effect than VP-16 on the breakage-reunion equilibrium of topoisomerase II and DNA. The stronger inhibition of the religation of the second strand observed with VPQ suggests it interacts asymmetrically with the two homodimers of topoisomerase II bound to DNA. © 1997 Academic Press

Eukaryotic topoisomerase II relaxes supercoiled DNA by passing the DNA helix through a reversible double strand break. This step is preceded by the formation of a transient DNA-protein complex, anchored by covalent phosphotyrosyl bonds between the active site of the homodimeric enzyme and the 5′ - DNA termini of the cleaved DNA strands [1]. When the DNA-topoisomerase II cleavable complex is trapped on the DNA by inhibitors, normal DNA metabolism such as transcription and replication is inhibited and cytotoxicity ensues. The podophyllotoxin derivative, etoposide (VP-16) inhibits the DNA rejoining steps during topoisomerase processing and has found wide applications as an anticancer agent [2]. The drug alters both the pre- and post-strand passage cleavage/religation equilibrium of topoisomerase II with DNA, resulting in accumulation of covalent DNA-protein complexes, which are reversible following drug removal [3-5].

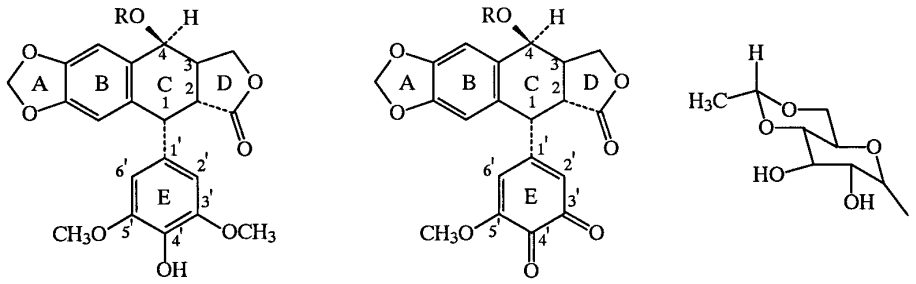
[1] Author for correspondence.

Etoposide (VP-16) can undergo oxido-reductive transformations in cells. Two major pathways have been identified: *O*-demethylation mediated by P450 dependent monooxygenases [6], and one- or two-electron oxidation catalyzed by some peroxidases and tyrosinase [7,8]. Both types of enzymatic transformations affect the pendant dimethoxyphenolic group (E-ring) of the drug, leading to formation of an *ortho*-quinone (VPQ) as a final product (Scheme I). The latter may be converted in cells to the semi-quinone radical and catechol, and/or it may react with nucleophilic groups of amino acids to inactivate sensitive enzymes [9-11]. During the peroxidative metabolism of etoposide a phenoxyl free radical is also formed [12]. All these reactive products probably affect the cytotoxic potential of the drug as evidenced by the ability of the *ortho*-quinone and the semi-quinone radical to induce DNA breaks and adducts *in vitro* [9,13], and the enhancement or suppression of etoposide toxicity by treatments that affect drug metabolic transformations [14,15]. It is however not known if the E-ring altered etoposide metabolic products are still capable of trapping the topoisomerase-DNA cleavable complex. Analysis of the effects of the *ortho*-quinone VP-16 derivative on topoisomerase processing is the subject of the present study.

MATERIALS AND METHODS

Reagents. Supercoiled DNA, pBR 322 (~ 30 superhelical turns per molecule), which contained ~ 15-18 % nicked circle DNA was purchased from Pharmacia (Upsala) and used as supplied. Purified human type II topoisomerase (p170 form, 2 U/μl) was obtained from TopoGen, Inc. (Columbus, Ohio). Etoposide (VP-16) was purchased from Sigma (St. Louis, MO) and dissolved in DMSO. The *ortho*-quinone derivative (VPQ) of etoposide was synthesized by controlled potential electrolysis of VP-16 at a Pt-gauze electrode in the absence of oxygen [16]. The pink-colored product was purified by liquid chromatography, recrystallized twice from chloroform and lyophilized. The structure and the purity were confirmed by IR spectroscopy (carbonyl bands at 1627, 1661, and 1709 cm^{-1}), mass-spectrometry and HPLC chromatography.

0006-291X/97 $25.00



SCHEME I. Structural formula of etoposide (VP-16) and its *ortho*-quinone derivative (VPQ).

Topoisomerase II mediated DNA cleavage. DNA cleavage reactions typically contained 6 U of purified topoisomerase II (approx. 10 nM) and 100 ng pBR 322 (~ 1 μM) per 20 μl sample in a cleavage buffer (10 mM Tris, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$ and 0.5 mM ATP). This enzyme activity assay (in the presence of Mg^{2+} and ATP) allows monitoring of the effects of VP-16 and VPQ on the complete cycle of the topoisomerase II mediated process of relaxation of supercoiled DNA, which includes the events of DNA binding, cleavage, strand-passage, religation and enzyme turnover. The complex was formed by mixing the enzyme and DNA on ice and the reaction was incubated at 37°C for different times. Unless stated otherwise, the inhibitors (VPQ and VP-16 in DMSO) were added to the already existing complex (before sample transfer to 37°C). DMSO (2.5 % v/v) was also present in control samples, although at this concentration it did not affect topoisomerase II activity. Cleavage products were trapped with 2 μl of 10 % SDS, and samples were incubated for an additional 5 min at 37°C. After addition of EDTA and NaCl, samples were treated with proteinase K (0.8 mg/ml final concentration) for 2 h at 55°C. The reversibility of the formation of the cleavable complex following chelation of divalent metal ions was assayed by the addition of 2 μl of 100 mM EDTA before trapping of topoisomerase II/DNA complexes with SDS [17], while keeping samples at 37°C. To prevent recleavage, 1 μl of 4M NaCl was also added with the EDTA.

Samples were subjected to electrophoresis in 1.3 % agarose in the presence of 0.7 μg/ml ethidium bromide at 2 V/cm for 18 h. Since agarose electrophoresis was performed in the presence of ethidium bromide, the completely relaxed closed circular (RLX) DNA migrated faster than supercoiled (SC) DNA, followed by the linear (LNR) and nicked circle (NC) DNA forms. Under these conditions, topoisomers of intermediate superhelicity were also resolved when the average change in the linking number was ≤10-20 % (PRLX). Negatives of gel photographs were scanned and DNA bands were quantitated using NIH Image (version 1.58) computer software together with appropriate calibration curves.

RESULTS AND DISCUSSION

The gel photograph presented in Fig. 1 shows the effects of different VPQ concentrations on the processing of supercoiled pBR 322 DNA by topo II. VPQ has a potent effect on the DNA cleavage/religation equilibrium resulting in increased formation of enzyme-mediated nicked forms of supercoiled DNA (linear and nicked circular). Similar effects are well known for the parent drug, VP-16 [3-5]. The relative changes in the levels of linear and nicked circular DNA (percent of total) as a function of VPQ concentration are shown in Fig. 2. Formation of linear DNA (i.e. trapping of the double-stranded cleavable complex) is enhanced progressively up to VPQ concentrations of approximately 25 μM and thereafter declines and attains a steady state level between 60 and 125 μM. At the same time, single strand breaks accumulate monotonously with increasing drug concentration. At VPQ concentrations greater than 25-30 μM, no relaxed DNA is observed (Fig. 1); instead, partially relaxed DNA molecules (topoisomers with an intermediate superhelicity) are present (lines 6 and 7, Fig. 1). Relaxed and partially relaxed DNA forms vanish altogether with further increases in VPQ concentration (lanes 8 and 9). These data indicate that, in addition to the trapping of the topoisomerase-DNA cleavable complex (i.e. by interfering with the strand religation steps), VPQ is able to

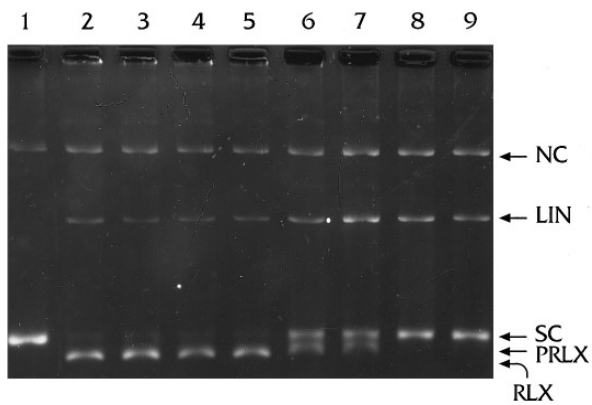


FIG. 1. Reaction products of topoisomerase II-catalyzed pBR322 relaxation in the presence of increasing concentrations of VP-16 *ortho*-quinone derivative (VPQ). Lanes: (1), 100 ng pBR 322; (2), 6 U topoisomerase II/DNA (no drug, control); and (3) - (9), topoisomerase II-DNA in the presence of 0.5 (3), 1.25 (4), 2.5 (5), 12.5 (6), 25 (7), (8) 62.5, and 125 μM VPQ. Reaction incubation for 8 min at 37°C. The positions of completely relaxed (RLX), supercoiled (SC), linear (LNR), nicked circular (NC), and partially relaxed (topoisomers with intermediate superhelicity (PRLX)) are indicated.

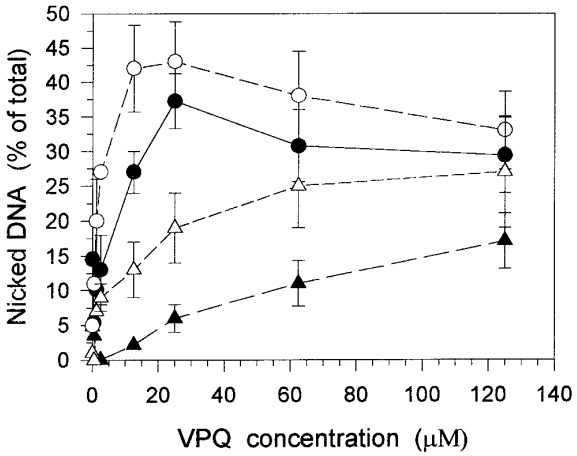


FIG. 2. Graphical representation of the accumulation of nicked DNA forms (as % of total DNA) with the increase VPQ concentration (data obtained by scanning gel photographs): (○) linear and (△) nicked circle DNA. Amount of nicked circle DNA present in pBR322 itself (~18%) was subtracted. Incubation of the complex in the presence of VPQ was performed at 37°C for 8 min (filled symbols) and for 30 min (opened symbols). Averaged data (± S.D.) from 3 independent experiments.

affect other events in topoisomerase processing (binding, cleavage and/or strand passage). To elucidate these possibilities, we performed experiments where VPQ was either preincubated with the protein for different periods before the addition of DNA, or the incubation time of VPQ with the already formed complex was varied. Increasing the preincubation time of the protein with VPQ up to 30 min before the addition of DNA had little effect on the overall inhibition of DNA processing (not shown). Incubation of VPQ with DNA alone, even at elevated temperatures, did not result in detectable changes in the DNA migration pattern. Increasing the incubation time of the VPQ with the complete reaction mixture (i.e. under conditions where most of the topo II should be bound to the DNA), augmented the levels of protein-related nicked DNA forms (i.e. an increase in single stranded breaks, Fig. 2). These data indicate that VPQ probably does not affect topoisomerase binding to DNA, but exerts its action primarily on the bound enzyme and/or on the DNA-protein complex.

Enzyme-mediated DNA breaks resulting from trapping of the cleavable complex by etoposide have been shown to be reversible, i.e. cleaved DNA strands can be religated upon addition of salt, EDTA, or by dilution, or heating [4,17]. Experiments were performed in order to determine whether the VPQ enhanced formation of DNA breaks was also reversible. The enzyme was allowed to establish equilibrium with DNA in the presence of 25 μM VPQ and the complex was incubated for 30 min at 37°C. The reaction was stopped by the addition EDTA/NaCl prior to the addition of SDS. As seen in lanes 3 and 4 (Fig. 3), drug-enhanced double-stranded DNA breaks were reversed. However, this was paralleled by a significant increase in the number of single-stranded breaks. It is noteworthy that this procedure also resulted in formation of additional slow-moving bands, tentatively assigned to partially digested DNA-protein complexes (lane 4, Fig. 3, arrows). The reversibility of the DNA breaks after treatment with VPQ was compared with the reversibility of the breaks induced by VP-16 under the same conditions. Graphical presentation of the results is given in Fig. 4. For both drugs, a similar degree of removal of double-stranded DNA breaks was observed, however this was accompanied by accumulation of more single-stranded breaks in the case of VPQ. While religation initiated by metal ion depletion in the presence of VP-16 decreases the amount of supercoiled and increases the amount of relaxed DNA, in the presence of VPQ a significant augmentation of the amount of supercoiled DNA was primarily observed. The latter indicates that under these conditions VPQ is a stronger inhibitor and traps the cleavable complex preferentially before the strand-passage event. Another important result is that while in the presence of any of the drugs, the first DNA strand of the complex is effectively resealed (elimination of double-stranded DNA breaks), in the presence of VPQ the rate of religation of the second strand is strongly attenuated (increased accumulation of single-stranded DNA breaks), as compared to VP-16. This specific difference indicates that VPQ may induce additional chemical modifications of the DNA bound enzyme which result in a partial impairment of the topoisomerase homodimer while bound to DNA (i.e. one of the subunits becomes less competent to reseal the cleaved DNA strand).

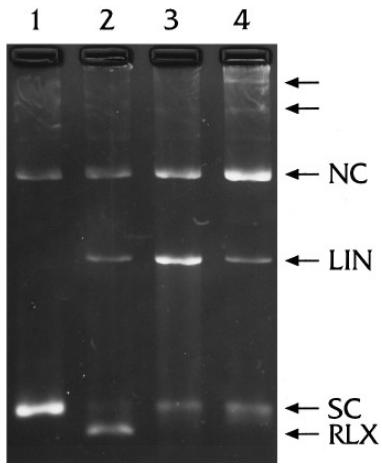


FIG. 3. Effect of the complexation of divalent metal ions by EDTA on the levels of DNA breaks mediated by topoisomerase in the presence of VPQ. Lanes: (1), DNA alone; (2), DNA/topoisomerase; and (3) and (4), DNA/topoisomerase incubated in the presence of 25 μM VPQ for 30 min. In lane (3), SDS was added prior to EDTA; in lane (4), EDTA was added prior to SDS.

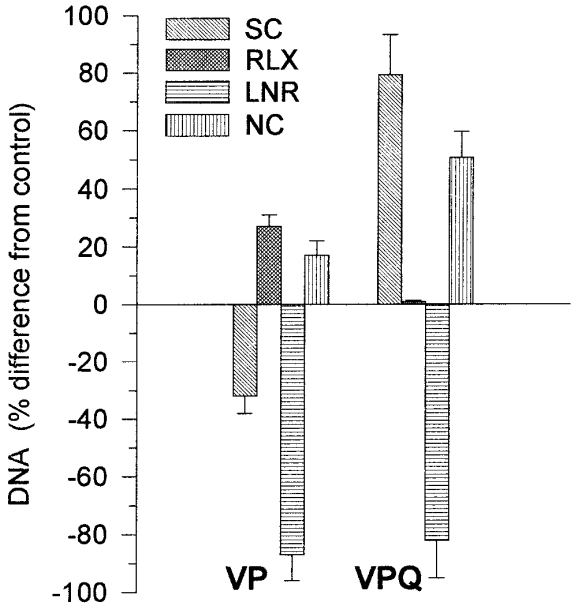


FIG. 4. Comparison of the effects of reversibility of DNA breaks induced by topoisomerase II in the presence of etoposide (VP-16) and its *ortho*-quinone derivative (VPQ). Relative amounts of different DNA forms (as indicated) were obtained after scanning gel photographs. In every case data were calculated as % difference from control. Samples where DNA/topoisomerase complexes in the presence of drugs were denatured by SDS prior to the addition of EDTA were used as controls.

CONCLUSIONS

The following conclusions about the inhibitory activity of the *ortho*-quinone derivative (VPQ) of the antitumor drug etoposide VP-16 against topoisomerase II and its DNA-cleavable complex can be drawn: (i) Both the parent compound, etoposide, and VPQ trap the protein-DNA cleavable complex. This is an important finding in view of structure-activity relationships and indicates that methoxy groups on the E-ring are not a prerequisite for the drug to act as a topoisomerase/DNA complex poison. Our data imply also that VPQ affects more effectively the breakage-reunion equilibrium of topoisomerase with DNA than does VP-16, and possibly inhibits strand passage; (ii) In addition, VPQ is unlikely to inactivate the enzyme itself, but presumably acts chemically within a specific site of the enzyme-DNA complex when the protein adopts a specific conformation upon DNA binding. The stronger inhibition of the religation of the second DNA strand implies asymmetric interactions (conformations) of the two homodimeric units of topoisomerase II while bound to DNA. In this study mechanistic aspects were not addressed, but nucleophilic addition reactions (formation of covalent adducts to specific sulfhydryl, or amino groups of the enzyme) and/or free radical-mediated oxidation reactions (semi-quinone radical-mediated) could be involved. Further experimental work is required to elucidate the detailed mode of action of VPQ on the topoisomerase II-DNA complex at the molecular and cellular levels.

ACKNOWLEDGMENTS

Supported by the Medical Research Council of Canada. T.G.G. is grateful to Dr. V. E. Kagan for his continued interest in this work.

REFERENCES

1. Wang, J. C. (1996) *Annu. Rev. Biochem.* **65,** 635–692.
2. Liu, L. F. (1990) *in* DNA Topology and its Biological Effects (Cozzarelli, N. R., and Wang, J. C., Eds.), pp. 371–389, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3. Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M., and Liu, L. F. (1984) *J. Biol. Chem.* **259,** 13560–13566.
4. Hsiang, Y.-H., and Liu, L. F. (1989) *J. Biol. Chem.* **264,** 9713–9715.
5. Robinson, M. J., and Osheroff, N. (1991) *Biochemistry* **30,** 1807–1813.
6. van Maanen, J. M. S., de Vries, J., Pappie, D., van den Akker, E., Lafleur, M. V. M., Retèl, J., van der Greef, J., and Pinedo, H. M. (1987) *Cancer Res.* **47,** 4658–4662.
7. Haim, N., Nemec, J., Roman, J., and Sinha, B. K. (1987) *Cancer Res.* **47,** 5835–5840.
8. Kagan, V. E., Yalowich, J. C., Day, B. W., Goldman, R., Gantchev, T. G., and Stoyanovsky, D. A. (1994) *Biochemistry* **33,** 9651–9660.
9. van Maanen, J. M. S., Lafleur, M. V. M., Mans, D. R. A., van den Akker, E., de Ruiter, C., Kootstra, P. R., Pappie, D., de Vries, J., Retèl, J., and Pinedo, H. M. (1988) *Biochem. Pharmacol.* **37,** 3579–3589.
10. Kalyanaraman, B., Premovic, P. I., and Sealy, R. C. (1987) *J. Biol. Chem.* **262,** 11080–11087.
11. Mans, D. R. A., Lafleur, M. V. M., Westmijze, E. J., Horn, I. R., Bets, D., Schuurhuis, G. J., Lankelma, J., and Retèl, J. (1992) *Biochem. Pharmacol.* **43,** 1761–1768.
12. Gantchev, T. G., van Lier, J. E., Stoyanovsky, D. A., Yalowich, J. C., and Kagan, V. E. (1994) *in* Methods in Enzymology, (Packer, L., Ed.), Vol. 234, pp. 631–642, Academic Press, San Diego.
13. Mans, D. R. A., Retèl, J., van Maanen, J. M. S., Lafleur, M. V. M., van Schaik, M. A., Pinedo, H. M., and Lankelma, J. (1990) *Br. J. Cancer* **62,** 54–60.
14. Gantchev, T. G., Brasseur, N., and van Lier, J. E. (1996) *Br. J. Cancer* **74,** 1570–1577.
15. Gantchev, T. G., and Hunting, D. J. (1997) *Anti-Cancer Drugs* **8,** 164–173.
16. Holthuis, J. J. M., van Oort, W. J., Römkens, F. M. G. M., and Renema, J. (1985) *J. Electroanal. Chem.* **184,** 317–329.
17. Osheroff, N. (1989) *Biochemistry* **28,** 6157–6160.