Eur J Cancer, Vol. 26, No. 5, pp. 590–593, 1990.
Printed in Great Britain

0277–5379/90$3.00 + 0.00

Structure–Activity Relations, Cytotoxicity and Topoisomerase II Dependent Cleavage Induced by Pendulum Ring Analogues of Etoposide

Birandra K. Sinha, Pedro M. Politi, Helen M. Eliot, Donna Kerrigan and Yves Pommier

The cytotoxicity of etoposide and its analogues, dihydroxy (DHVP), *o*-quinone (VP-Q) and *o*-methyl (VP-OMe), was evaluated in human breast (MCF-7) and HL60 tumour cells. Although less potent than etoposide, both DHVP and VP-Q were cytotoxic to these cells. However, VP-OMe was inactive. Studies with purified topoisomerase II showed that the intensity of DNA cleavage and the pattern of cleavage were similar for DHVP, VP-Q and etoposide. In contrast, the VP-OMe failed to induce DNA cleavage, indicating that the presence of 4'-OH is essential for metabolism, induction of topoisomerase II-mediated DNA cleavage and cytotoxicity of etoposide and its analogues.
Eur J Cancer, Vol. 26, No. 5, pp. 590—593, 1990.

INTRODUCTION

CELLULAR DNA damage caused by etoposide [1] may account for its clinical activity. Loike and Horwitz [2] have demonstrated that the DNA breaking activity induced by etoposide required the presence of cellular components and topoisomerase II was later proposed as the intracellular target responsible for DNA damage [3–5]. Microsomal cytochrome P-450 and peroxidases (horseradish or prostaglandin synthase) metabolize etoposide to its *o*-dihydroxy (DHVP) and *o*-quinone derivatives (VP-Q) [6–8]. Reactive intermediates are formed during etoposide activation that become bound covalently to proteins and DNA. This metabolism required a free hydroxyl at the 4' position group in the E-ring, as the fully O-methylated derivative (VP-OMe) neither formed the phenoxy radical nor produced any metabolites. Therefore activation may be important for the activity of the parent drug and VP-Q and DHVP may interact with topoisomerase II, induce DNA breakage and thus possess antitumour activity of their own [7, 9]. We have examined the cytotoxicity of these pendulum ring analogues of etoposide in two human tumour cell lines, including their ability to induce topoisomerase II dependent DNA cleavage.

MATERIALS AND METHODS

Etoposide, DHVP and VP-Q were given by Bristol-Myers, Wallingford, Connecticut. VP-OMe was prepared by a diazomethane reaction by Dr J. Nemec of Frederick NCI-Cancer Facility, Frederick, Maryland. The chemical purity and structures were checked [7].

Correspondence to B.K. Sinha at Bldg-10, Rm. 6N-119, NCI, NIH, Bethseda, MD 20892, U.S.A.
B.K. Sinha, P.M. Politi and H.M. Eliot are at the Biochemical Pharmacology Section, Medicine Branch and D. Kerrigan and Y. Pommier are at the Laboratory of Molecular Pharmacology, National Cancer Institute, National Institutes of Health, Bethseda, Maryland, U.S.A.

The human breast tumour cell line (MCF-7) was grown in improved modified essential medium (IMEM) containing 5% foetal bovine serum, and 50 μg/ml gentamicin. The human promyelocytic leukaemia cell line (HL-60) was grown in suspension culture in RPMI 1640 supplemented with 10% foetal bovine serum, and 50 μg/ml gentamicin. Cells used in these studies were in exponential growth phase.

The cytotoxicity of etoposide and the analogues was assessed by colony formation assay. For MCF-7 cells, the cells were trypsinized and 500 cells were seeded in triplicate in six-well Linbro dishes in 2 ml medium and allowed to attach for 18 h at 37°C. The medium was removed and the drugs, dissolved in dimethylsulphoxide (DMSO) and diluted with medium without serum, were added and incubated for 1 h. The drugs were removed by washing the cells with medium. The cells were supplemented with the complete medium and allowed to grow for 12–14 days, stained with 0.5% methylene blue in 50% methanol, and the colonies counted. For HL-60 cells, 400 cells were seeded in triplicate in sterile tubes containing colony stimulating factors (Gibco), RPMI, foetal calf serum and 0.1% soft agar in 10 ml. Colonies were counted after 14 days. The highest concentration of DMSO used to dissolve the drugs was less than 1% and controls contained identical concentrations of DMSO. The cloning efficiency was 40–50% for MCF-7 and 20–30% for HL60 cells under our experimental conditions.

To assess topoisomerase II mediated DNA cleavage, SV40 DNA, restriction enzymes, polynucleotide kinase, calf alkaline phosphatase and agarose were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Polyacrylamide and [^{32}P]ATP were obtained from Bio-Rad (Richmond, California) and New England Research Products (Boston, Massachusetts), respectively. Type II DNA topoisomerase was purified from mouse leukaemia (L1210) cells [10]. Enzyme purity was checked by silver staining of sodium dodecylsulphate–polyacrylamide gels showing a prominent 170 kDa band and fainter bands between 170 and 150 kDa [11].

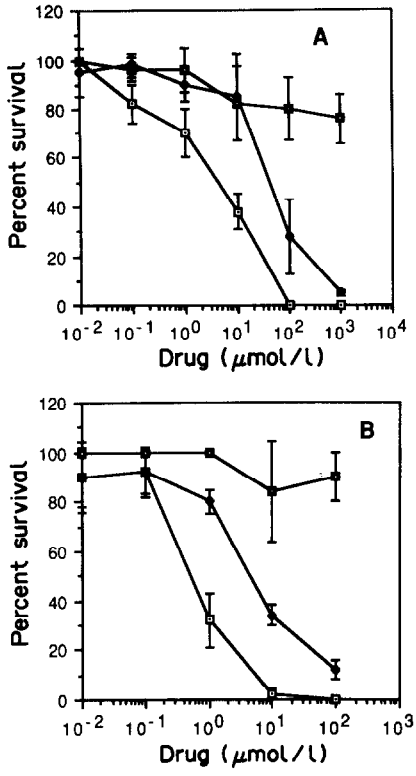

Fig. 1. Cytotoxicities of etoposide (□—□), VP-Q (◆—◆) and VP-OMe (■—■) in (A) MCF-7 cells and (B) HL60 cells after 1 h drug exposure (mean ± S.D.).

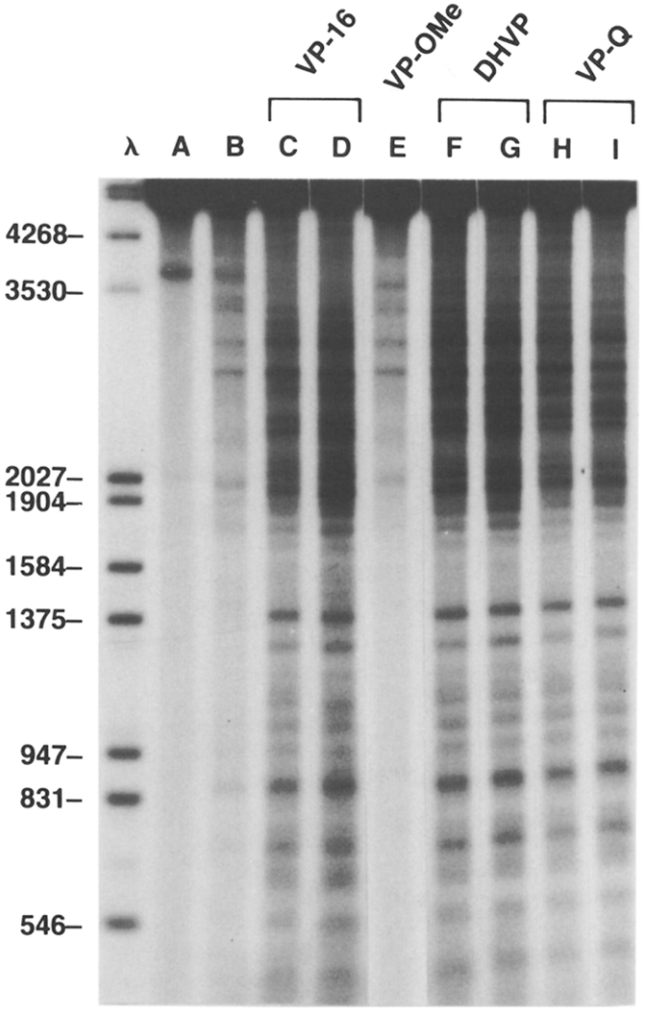

Fig. 2. Cleavage of SV40 DNA by topoisomerase II in presence of etoposide and its analogues. A = control DNA, B = DNA reacted with L1210 topoisomerase II in absence of drugs, C and D = etoposide 5 and 20 μmol/l, E = VP-OMe 50 μmol/l, F and G = DHVP 5 and 20 μmol/l, and H and I = VP-Q 5 and 20 μmol/l. *Hind* III/*Eco*R I lambda DNA markers: size of fragments indicated on left. VP-16 = etoposide.

One unit of topoisomerase was defined as the amount of enzyme yielding 90% DNA relaxation in 30 min at 37°C.

^{32}P end-labelled SV40 DNA was prepared [4], and the two restriction enzymes used to produce the two end-labelled fragments of 5200 base pairs and 50 base pairs were *Ban* I/*Hpa* II and *Acc* I/*Eco*R I. Reactions were done as described [4] and at the end of electrophoresis, gels were dried on to 3 MM filter sheets and autoradiographed with Kodak XAR 5 film (Eastman Kodak, Rochester, New York).

The partition coefficients (octanol:buffer) of etoposide, DHVP, VP-Q and VP-OMe were determined by adding the drugs dissolved in polyethylene glycol (final volume 0.2%) to 1 ml octanol (solution A) and then preparing a mixture containing 0.5 ml octanol and 0.5 ml of phophate-buffered saline (solution B). After mixing, samples were spun at room temperature for 1 min. Aliquots (20 μl) were taken from the initial mixture after spinning, and from both aqueous and octanol phase, and injected into a reverse phase high-performance liquid chromatography system with a mobile phase of 60% methanol and 40% water [5]. The solvent was pumped isocratically at 1 ml/min, through a Bondapak phenyl column. Absorbance was monitored at 277 nm. The partition coefficient was calculated as the percentage peak height ratio (solution B/solution A) for at least three experiments.

RESULTS

While DHVP and VP-Q showed modest activity in both MCF-7 and HL60 cells, etoposide was significantly more toxic to these cells (Fig. 1). In contrast, VP-OMe had little or no cytotoxicity in these cell lines. DHVP was more cytotoxic (2-fold) than VP-Q. Etoposide, DHVP and VP-Q were more toxic (10-fold) to HL60 cells than to MCF-7 cells.

Because both the *o*-derivatives of etoposide, DHVP and VP-Q, were active in killing HL60 and MCF-7 tumour cells and VP-OMe was inactive, we compared the activity of these analogues in inducing DNA cleavage in the presence of purified topoisomerase II. Both DHVP and VP-Q induced topoisomerase II mediated cleavage of ^{32}P end-labelled *Acc* I/*Eco*R I SV40 DNA while VP-OMe was inactive (Fig. 2). The intensity of DNA cleavage was similar for DHVP and etoposide at equal concentrations, and was slightly less for VP-Q. This was analysed further by comparing etoposide and its quinone metabolite, VP-Q, in *Ban* I/*Hpa* II DNA (Fig. 3). More cleavage was induced by etoposide 10 μmol/l than by the same concentration of VP-Q, and depletion of longer DNA fragments was detected with

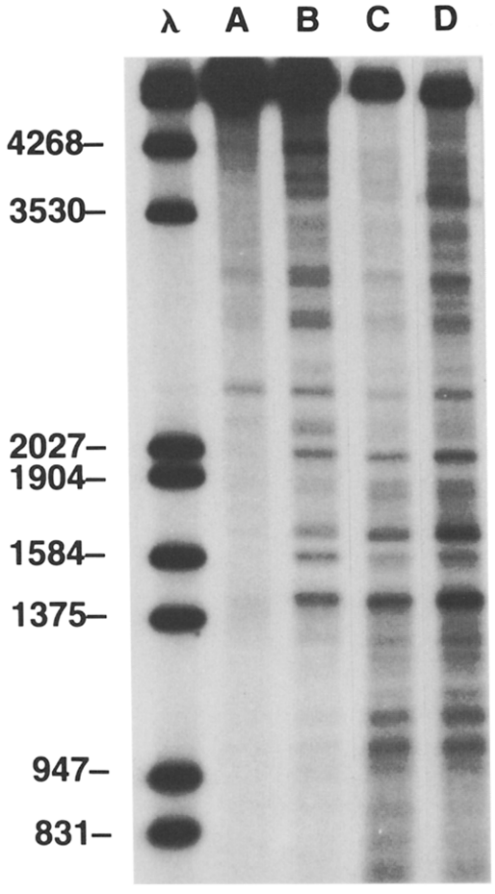

Fig. 3. Topoisomerase II mediated DNA cleavage induced by etoposide and VP-Q. Lane A = control DNA, B = DNA reacted with topoisomerase II alone, C = etoposide 10 μmol/l D = VP-Q, and 10 μmol/l. *Hind* III/*Eco*R I lambda DNA markers: size of fragments indicated on left.

etoposide (lane C) than with VP-Q (lane D). Distribution patterns of the DNA cleavage sites, however, were similar in the two DNA fragments analysed (Figs 2 and 3).

DHVP and VP-Q were more polar than the parent drug. Furthermore, there was a positive correlation between their respective IC_{50} (concentration of the drug required to inhibit cell growth by 50%) values and their partition coefficients (Fig. 4).

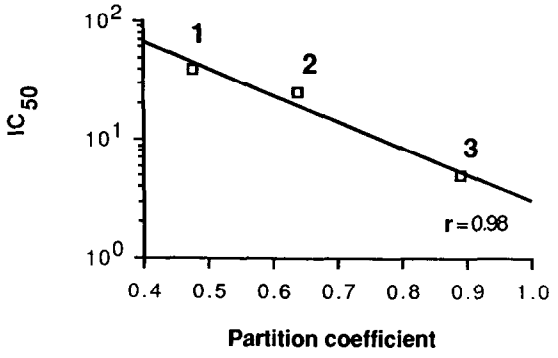

Fig. 4. Relation between cytotoxicity (in MCF-7 cells) and octanol/water partition coefficient for VP-Q (1), DHVP (2) and etoposide (3). IC_{50} in μmol/l.

DISCUSSION

The reasons for the enhanced toxicity in HL60 cells are not clear. However, our previous studies showed that significantly more etoposide is accumulated and retained in HL60 cells than in MCF-7 cells at equal extracellular drug concentrations [12]. Moreover, it is possible that myeloperoxidase, present in HL60 cells, may lead to the increased formation of toxic metabolites. Peroxidases have been shown to activate etoposide to its phenoxy radical and to form VP-Q [13]. The two metabolites of etoposide, VP-Q and DHVP, formed as a result of oxidative metabolism, can inhibit purified topoisomerase II in a manner similar to the parent compound. Furthermore, no differences were observed in distribution patterns of DNA cleavage compared with the parent drug. DHVP and VP-Q interacted with the same binding sites in the topoisomerase II-DNA complexes as did etoposide. In contrast to VP-Q and DHVP, VP-OMe failed to induce topoisomerase II mediated DNA breakage. Our data confirm the earlier observations of Long *et al.* [3] who showed that the free hydroxyl group at the 4′-position is essential for DNA breakage activity in human lung adenocarcinoma cells and indicate that the 4′-OH group of etoposide is also essential for recognizing the drug binding site(s) of topoisomerase II-DNA complex.

These results also indicate that etoposide and DHVP are cytotoxic, supporting the idea that the metabolism of etoposide yields active derivatives. Furthermore, a free 4′-OH in the E-ring is essential for the formation of the phenoxy radical (and the *o*-metabolites), topoisomerase II inhibition and cytotoxicity since VP-OMe neither forms a radical nor is it metabolized to DHVP and VP-Q, active against purified topoisomerase II or cytotoxic in the two cell lines we studied. Thus, using two different and independent assays, we showed that the biological activity of etoposide and its *o*-analogues requires this.

Although VP-Q and DHVP had antitumour activity, they were less potent than the parent drug despite having similar topoisomerase II induced DNA breakage activity. This was not unexpected since these compounds are polar and thus taken up slowly by cells. This is in agreement with our partition coefficient study which showed a good correlation with cytotoxicity. Since both *o*-metabolites of etoposide were as efficient as the parent drug in inducing topoisomerase II mediated DNA cleavage *in vitro*, the decreased cytotoxicity of these analogues may reflect decreased cellular uptake as a result of the increased polarity of these analogues. Since both DHVP and VP-Q bind indiscriminantly to proteins and cellular macromolecules more than etoposide [6, 14], we used the partitioning of these analogues in *n*-butanol and water as a measure of polarity. It is therefore possible that the intracellular metabolism of etoposide to VP-Q and DHVP has a role in the cytotoxicity of etoposide. The mechanism of action of VP-Q and DHVP could then involve binding to topoisomerase II–DNA complex and induction of the formation of protein-linked DNA breaks. In addition, it is possible that covalent binding to DNA and to the sulphydryl groups of other critical proteins by the *o*-quinone and DHVP may inhibit enzymatic and cellular functions. These events may ultimately cause cell death.

1. O'Dwyer PJ, Leyland-Jones B, Alonso MT, Marsoni S, Witte RE. Etoposide (VP-16-213): current status of an active anticancer drug. *N Engl J Med* 1985, **312**, 692–700.
2. Loike JD, Horwitz SB. Effects of VP-16-213 on the intracellular degradation of DNA in HeLa cells. *Biochemistry* 1976, **15**, 5443–5448.

3. Long BH, Musial ST, Brattain MG. Comparison of cytotoxicity and DNA breakage activity of congeners of podophyllotoxin including VP-16-213 and VM-26: a quantitative structure–activity relationship. *Biochemistry* 1984, **23**, 1183–1188.
4. Pommier Y, Kohn KW. Topoisomerase II inhibition by antitumor intercalators and demethylepipodophyllotoxins. In: Glazer RI, ed. *Development in Cancer Chemotherapy*. Boca Raton, CRC Press, 1989, Vol. II, 175–195.
5. Sinha BK, Haim N, Dusre L, Kerrigan D, Pommier Y. DNA strand breaks produced by etoposide (VP-16,213) in sensitive and resistant human breast tumor cells: implications for the mechanism of action. *Cancer Res* 1988, **48**, 5096–5100.
6. Haim N, Nemec J, Roman J, Sinha BK. *In vitro* metabolism of etoposide (VP-16-213) by liver microsomes and irreversible binding of reactive intermediates to microsomal proteins. *Biochem Pharmac* 1987, **36**, 527–536.
7. Haim N, Nemec J, Roman J, Sinha BK. Peroxidase-catalyzed metabolism of etoposide (VP-16,213) and covalent binding of reactive intermediates to cellular macromolecules. *Cancer Res* 1987, **47**, 5835–5840.
8. van Maanen JMS, de Vries J, Pappie D *et al.* Cytochrome P-450-mediated O-demethylation: a route in the metabolic activation of etoposide (VP-16,312). *Cancer Res* 1987, **47**, 4658–4662.
9. van Maanen JMS, Retel J, de Vries J, Pinedo HM. Mechanism of action of antitumor drug etoposide: a review. *J Natl Cancer Inst* 1988, **80**, 1526–1533.
10. Minford J, Pommier Y, Filipski J *et al.* Isolation of intercalator-dependent protein-linked DNA strand cleavage activity from cell nuclei and identification as topoisomerase II. *Biochemistry* 1986, **25**, 9–16.
11. Liu F, Miller KG. Eukaryotic DNA topoisomerases: two forms of type I DNA topoisomerases from HeLa cell nuclei. *Proc Natl Acad Sci USA* 1981, **78**, 3487–3491.
12. Politi PM, Sinha BK. Role of differential drug uptake, efflux, and binding of etoposide in sensitive and resistant human tumour cell lines: implications for the mechanisms of drug resistance. *Mol Pharmac* 1989, **35**, 271–278.
13. Kalyanaraman B, Nemec J, Sinha BK. Characterization of free radicals produced during oxidation of etoposide (VP-16) and its catechol and quinone derivatives. An ESR study. *Biochemistry* 1989, **28**, 4839–4846.
14. van Maanen JMS, Lafleur MV, Mans DRA *et al.* Effects of the *ortho*-quinone and catechol of the antitumor drug VP-16-213 on the biological activity of single-stranded and double-stranded ϕX174 DNA. *Biochem Pharmac* 1988, **37**, 3579–3589.

Eur J Cancer, Vol. 26, No. 5, pp. 593–596, 1990.
Printed in Great Britain

0277–5379/90$3.00 + 0.00

Early Serum CA125 Response and Outcome in Epithelial Ovarian Cancer

Charles W.E. Redman, George R. Blackledge, Krystyna Kelly, Judith Powell, Edward J. Buxton and David M. Luesley

The prognostic value of serum CA125 levels before and after two courses of chemotherapy was assessed in 50 patients with advanced epithelial ovarian cancer. Patients with serum CA125 values below 35 U/ml after two courses were significantly more likely to achieve complete remission and had a significantly longer median survival. In multivariate analysis, serum CA125 levels after two courses were the most important independent prognostic factor: it was possible to predict survival status at 12 months with an overall accuracy of 93%. Serum CA125 can be used to evaluate quantitatively chemotherapeutic response and at an early stage classify patients into good and poor risk groups. Such an approach would facilitate the selection of appropriate therapy and could reduce toxicity.
Eur J Cancer, Vol. 26, No. 5, pp. 593—596, 1990.

INTRODUCTION

SERUM CA125 has been extensively studied in relation to the management of ovarian cancer [1–3]. Initial studies evaluated the correlation with observed response [4, 5], although false negatives in small volume disease are a problem. Used in this way CA125 assay has little value other than sparing some patients second-look surgery, which is itself questionable [6]. Several studies have evaluated early CA125 response to predict outcome [7–9]. Van der Burg *et al.* [9], using serial measurements, reported that patients with a CA125 half-life of less than 20 days had a significantly longer median survival compared with patients with a longer half-life. However, 40% (approximately half of which had FIGO stage I disease) of the study group had no macroscopic postoperative disease and these findings are not necessarily relevant to patients with residual disease. Lavin *et al.* [7] correlated serum CA125 levels at 3 months with response at second-look surgery: all patients with levels greater than

Correspondence to: C.W.E Redman, Department of Obstetrics and Gynaecology, North Staffordshire Maternity Hospital, Stoke-on-Trent, U.K.
C.W.E. Redman, E.J. Buxton and D.M. Luesley are at the Department of Obstetrics and Gynaecology, University of Birmingham and G.R. Blackledge, K. Kelly and J. Powell are at the West Midlands CRC Clinical Trials Unit, Queen Elizabeth Medical Centre, Birmingham, U.K.