
Bonnie Glisson, Radhey Gupta, Sherin Smallwood-Kentro, and Warren Ross

Departments of Pharmacology and Medicine, University of Florida College of Medicine, Gainesville, Florida 32610 [B. G., S. S.-K., and W. R.], and Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada [R. G.]

ABSTRACT

Recent evidence indicates that type II DNA topoisomerases mediate epipodophyllotoxin-induced DNA damage and may be intrinsic to the drug’s antitumor effects. Using an epipodophyllotoxin-resistant cell line, we have now further defined the relationship between DNA damage and cell death and delineated the significance of certain drug–enzyme interactions. When compared to wild-type cells, the mutant Chinese hamster ovary cell line, Vpm5−5, exhibits marked resistance to both the cytotoxic and DNA cleavage activities of etoposide (VP-16). Steady-state concentrations of radiolabeled VP-16 are identical in both cell lines. Catalytic activity in crude nuclear extracts from wild-type and Vpm5−5 cells is equal and is equally sensitive to inhibition by VP-16. However, using an assay that specifically measures generation of 5’ protein-linked breaks in 32P-labeled 3’ DNA, we have found that DNA cleavage activity in nuclear extract from the Vpm5−5 line is profoundly resistant to stimulation by VP-16. Further, a somatic cell hybrid line of Vpm5−5 cells and drug-sensitive EOT-3 cells exhibits recovery of VP-16 sensitivity in concert with reconstitution of DNA cleavage activity. These data indicate that stimulation of enzyme-mediated DNA cleavage, rather than loss of normal topoisomerase function, is responsible for epipodophyllotoxin-induced cytotoxicity.

INTRODUCTION

Evidence accrued from this laboratory (1, 2) and from others (3–5) is strongly supportive of the concept that the antitumor activity of the epipodophyllotoxins, as well as several diverse intercalating agents, is mediated through interaction of drug with a type II DNA topoisomerase. The most persuasive evidence for this is the recent demonstration that m-AMSA-induced, protein-associated DNA breaks created intracellularly can be immunoprecipitated with polyclonal antibody specific for type II topoisomerase (6).

Previous work with VP-16 has shown that treatment of whole cells and isolated nuclei induced not only single strand breaks in DNA but also DNA-protein cross-links and DNA double strand breaks (7). Further studies with isolated nuclei demonstrated that VP-16-induced DNA damage involved a temperature-sensitive interaction between drug and a heat-labile intra-nuclear component. In addition, drug activity required the presence of magnesium and was stimulated by ATP but not a nonhydrolyzable analogue (1). Separate experiments using a highly purified mammalian type II topoisomerase demonstrated that both VP-16 and VM-26 resulted in enhancement of enzyme-mediated DNA cleavage, an effect which was also stimulated by ATP (2). Unlike the intercalating agents which were also active in this assay, we found that [3H]VP-16 did not bind to DNA during exhaustive equilibrium dialysis (2). Thus, epipodophyllotoxins appear to induce DNA damage through a direct interaction with the enzyme or enzyme-DNA complex.

Mammalian type II topoisomerases are homodimeric proteins (M, 170 kilodaltons) whose roles in vivo are just now becoming understood (8, 9). In vitro, they are characterized by their ability to catalyze the topological passing of two double-stranded DNA segments by transiently introducing a reversible double strand break in one of the crossing segments. During this reaction, an enzyme subunit is covalently bound to the 5’ end of the nicked DNA (10). Drugs such as VP-16, which result in the accumulation of protein-linked breaks in DNA, appear to block reversibility of this reaction by inhibiting nick closure and, thereby, stabilizing the so-called “cleavable complex,” resulting in fragmentation of DNA (3, 11). It has also been observed that these drugs inhibit the enzyme’s strand-passing functions, i.e., catalytic activity (3, 11). However, the precise relationship of these two separate effects to eventual cell death remains unclear.

In the present paper, we have used a previously described epipodophyllotoxin-resistant CHO cell line, Vpm5−5 (12), to further define drug–enzyme interactions that are critical in cell death. The Vpm5−5 line is markedly resistant to the DNA cleavage activity of VP-16 as compared to WT cells. We have found that type II topoisomerase catalytic activity is identical in both cell lines. In addition, catalytic activity from both lines is equally sensitive to inhibition by VP-16. In contrast, DNA cleavage activity in crude nuclear extracts of the Vpm5−5 line is profoundly resistant to stimulation by VP-16 when compared to WT cells. Further, studies with a somatic cell hybrid line demonstrate that recovery of VP-16 sensitivity is accompanied by reconstitution of drug-mediated DNA cleavage activity. Our findings serve to further implicate formation of the cleavable complex as the lethal lesion induced in the cell by the epipodophyllotoxins.

MATERIALS AND METHODS

Chemicals. Cell culture medium, fetal calf serum, trypsin, and HBSS were purchased from Grand Island Biological Co. (Grand Island, NY). [14C]Thymidine (53 mCi/mmol) and [α-32P]ATP (3200 Ci/mmol) were obtained from ICN (Irvine, CA). [3H]Etoposide (200 mCi/mmol) was obtained from Oravec Biochemicals (Brea, CA) and was 92% pure by high pressure liquid chromatography. Etoposide was a gift from Bristol Laboratories (Syracuse, NY). m-AMSA was obtained from Sigma Chemical Co. (St. Louis, MO) except where specifically noted.

Cell Lines and Culture Techniques. CHO cells were grown in mono
Decatenation of kDNA was carried out by incubating serial dilutions of extract with 1 μg of kDNA in a final volume of 20 μl at 30°C for 30 min. Reaction was terminated by the addition of 5 μl of 2% SDS, 0.05% bromophenol blue, and 50% glycerol. Samples were then electrophoresed through 1% agarose in 90 mM Tris borate-2 mM EDTA (pH 8.0) at 2 V/cm for 18 h. After staining with ethidium bromide (5 μg/ml), gels were photographed under UV illumination.

Drug-stimulated DNA cleavage activity was assayed qualitatively by the generation of form III (linearized) DNA from supercoiled (form I) pBR322 DNA in a final volume of 50 μl for 15 min at 37°C. The reaction was terminated by the addition of SDS to 1% and proteinase K at 0.1 mg/ml. Samples were then analyzed by electrophoresis through 1% agarose in a manner identical to the one used in the decatenation assay.

Quantitative analysis of DNA cleavage activity was assayed using 32P end-labeled DNA restriction fragments and the SDS-KCl precipitation of topoisomerase II-DNA complexes as originally described by Liu (10).

Table I: [3H]VP-16 cellular concentrations in WT and VpmR-5 cells

<table>
<thead>
<tr>
<th>Intracellular [etoposide] (nM)</th>
<th>Extracellular [etoposide] (μM)</th>
<th>Intracellular [etoposide] (nM/g CHO cells, dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>12.5</td>
<td>6.0 ± 2.3</td>
</tr>
<tr>
<td>25</td>
<td>6.0 ± 2.6</td>
<td>5.6 ± 0.8</td>
</tr>
<tr>
<td>50</td>
<td>9.6 ± 2.6</td>
<td>8.4 ± 1.7</td>
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<tr>
<td>100</td>
<td>19.3 ± 3.9</td>
<td>17.8 ± 4.0</td>
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* Mean ± SD of three separate experiments.
RESULTS

In order to quantify the degree of resistance, we have defined it as the factor by which drug dose must be multiplied to produce equitoxic effects in the Vpm<sup>R</sup>-5 line when compared to WT cells. The 20-fold level of resistance of the Vpm<sup>R</sup>-5 line to the cytotoxic effects of VP-16 is demonstrated in Fig. 1 and is similar to that published by Gupta (12). Due to the high level of resistance of these cells to VP-16-induced DNA cleavage activity, a high sensitivity alkaline elution assay was necessary to compare single strand break frequency in WT and Vpm<sup>R</sup>-5 lines. This is represented in Fig. 2, where the level of resistance of the Vpm<sup>R</sup>-5 line is 30-fold. Single strand break frequency is expressed as the amount of radiation required to produce equivalent DNA damage.

To rule out resistance mediated by altered transport of drug alone, we determined the intracellular concentration of [H]-VP-16 after a 1-h drug exposure and found no significant differences between WT and Vpm<sup>R</sup>-5 cells (Table 1). We also observed no difference in residual drug concentrations after a 1-h drug washout period (data not shown).

Type II topoisomerase catalytic activity in crude 0.35 M NaCl nuclear extracts from both cell lines was assayed by decatenation of kDNA. In the absence of type II topoisomerase activity, the network DNA barely enters the gel and is seen as a thin band at the top of the gel. Upon decatenation into simple circles, the DNA migrates more rapidly and is visualized as a broad band at the bottom of the gel. Fig. 3 demonstrates that activity in nuclear extract from both lines is very nearly equipotent. We performed this assay repeatedly with different extracts and were unable to document any consistent or significant differences between activity from WT and Vpm<sup>R</sup>-5 cells. Interestingly, we also found that catalytic activity from both lines is very nearly equivalent to inhibition by VP-16 as shown in Fig. 4. Notably, this effect requires rather high doses of drug.

In contrast to the above findings, we found that DNA cleavage activity in extract from Vpm<sup>R</sup>-5 cells is quite resistant to stimulation by VP-16. This is illustrated in Fig. 5 where a gel cleavage assay, performed as described in “Materials and Methods,” is represented. In the absence of drug, extract converts supercoiled pBR322 (form I) DNA to its relaxed form (forms II and IV). Significant form III generation (linearized DNA), reflecting double strand breaks in the DNA, occurs at a VP-16 concentration as low as 5 μM with extract from WT cells. The same effect is seen with extract from Vpm<sup>R</sup>-5 cells only at very high doses of VP-16 in the range of 100–200 μM. Drug-stimulated DNA cleavage in this assay could not be demonstrated in the absence of ATP (data not shown).

In order to more precisely quantify this effect and, more importantly, to verify that we were measuring type II topoisomerase-mediated DNA cleavage specifically, we performed the SDS-KCl precipitation DNA cleavage assay with extract from both lines in the presence of VP-16 (Fig. 6). In this assay, DNA cleavage products linked to protein are precipitated in SDS-KCl solution. By using uniquely 3'-end-labeled [32P]DNA, only the DNA cleavage products covalently linked to protein at the 5' end are radiolabeled. Extract from the Vpm<sup>R</sup>-5 line exhibits an approximate 26-fold level of resistance to stimulation by VP-16 in this assay. This correlates well with the results of alkaline elution and clonogenic assays in whole cells. Controls with 2% DMSO, that DMSO concentration in samples treated with VP-16 (200 μM), and WT extract revealed no increase in precipitated [32P]DNA as compared to extract alone.

Further corroborative evidence that this in vitro assay is reflective of events in the cell comes from similar experiments with m-AMSA, another agent which stimulates type II topoisomerase-mediated DNA cleavage, as shown in Fig. 7. The degree of resistance of the Vpm<sup>R</sup>-5 extract to stimulation by m-AMSA is less marked than that to VP-16. In separate studies (21), we have found that the degree of cross-resistance of the Vpm<sup>R</sup>-5 line to m-AMSA-mediated cytotoxicity and strand breaking activity is similarly less profound.

DISCUSSION

The observation that epipodophyllotoxin resistance in the Vpm<sup>R</sup>-5 line is mediated by loss of drug-stimulated DNA cleavage activity is perhaps the strongest evidence yet to link DNA strand scission directly with antitumor effect. Clearly, our data are corroborative of other less direct evidence in the literature supporting a relationship between type II topoisomerase-mediated DNA cleavage and cytotoxicity. We have previously shown that disulfiram, a sulfhydryl blocking agent, is a potent inhibitor of VP-16-induced strand breaks and cytotoxicity over similar dose ranges (22). More recently, this same phenomenon has been observed with ethidium bromide, a weakly cytotoxic intercalating agent, which inhibits epipodophyllotoxin-induced DNA cleavage by a purified type II topoisomerase (23). Simultaneous exposure of whole cells to this agent and VP-16 resulted in inhibition of both DNA strand breaks and cytotoxicity in a dose-dependent fashion. Long et al., in studying several epipo-
LOSS OF DNA CLEAVAGE ACTIVITY WITH ACQUIRED RESISTANCE TO VP-16

Fig. 4. Effect of VP-16 on decatenation of kDNA. Numbers refer to [VP-16] in micromolar units. Extract dose was 2 μl in a final volume of 20 μl. CTL, v.

Fig. 5. Effect of VP-16 in stimulating DNA cleavage activity in crude nuclear extracts from WT and VpmR-5 cells. Numbers refer to VP-16 concentration in micromolar units. Extract dose was 10 μl in a final volume of 50 μl. CTL, v.

Fig. 6. Effect of VP-16 in stimulating type II topoisomerase-mediated DNA cleavage activity in WT and VpmR-5 extracts. One on the ordinate represents the number of 32P cpm precipitated by extract alone. Extract dose was 10 μl in a final volume of 50 μl. Points, mean of three separate experiments.

Fig. 7. Effect of m-AMSA on type II topoisomerase-mediated DNA cleavage activity in extracts from WT and VpmR-5 cells. Extract dose was 10 μl in a final volume of 50 μl. Points, mean of two separate experiments.

Fig. 8. VP-16-induced single strand break (SSB) frequency in WT and M2J7 cells as measured by rad equivalents. Cells in monolayers were treated with drug for 1 h at 37°C, rinsed with cold PBS, and harvested by scraping in 1 ml of HBSS-0.02% EDTA. DNA was eluted at pH 12.1 at a speed of 0.16–0.20 ml/min with fraction intervals of 5 min and total elution time of 30 min. Points, mean of two separate experiments.

dophyllotoxin congeners, found that potency in inducing DNA strand scission in human lung adenocarcinoma cells was directly related to cytotoxic effects (24). In addition, our previous work comparing the activity of VP-16 with its congener, VM-26, has shown that VM-26 is 10-fold more potent in inducing cleavage of isolated DNA by purified enzyme, a finding that is well correlated with this agent's increased cleavage activity and cytotoxic effects in vivo (2).

Inhibition of type II topoisomerase strand-passing activity by drug appears unlikely to play a significant role in cytotoxicity as compared to drug-stimulated DNA cleavage by enzyme. That this was true had previously been suggested by work with congeners of intercalating agents, namely o- and m-AMSA, and ellipticine and 2-methyl-9-hydroxyellipticin (3, 5). Each pair of congeners has nearly equal capacity to inhibit catalytic activity of purified enzyme, yet o-AMSA and ellipticine are far less active in stimulating DNA cleavage and are much less cytotoxic than their respective partners. Ethidium bromide,
another intercalator which is only weakly cytotoxic and which blocks rather than stimulates type II topoisomerase-mediated DNA cleavage, is a very potent inhibitor of the enzyme's strand-passing activity (23).

Despite the use of crude nuclear extracts with admitted nuclelease and other nonhistone protein contamination, by using assays specific for type II topoisomerase, we have been able to show that resistance in the VpmR@5 line appears to be mediated by decreased sensitivity of enzyme-mediated cleavage of DNA to stimulation by drug. Thus, drug sensitivity must, in part, be dependent upon the ability of drug to stimulate cleavage of DNA by the enzyme. While defining the precise mechanism of resistance in the VpmR@5 line awaits purification of enzyme from both lines, it is our working hypothesis that the mutation in the VpmR@5 line has resulted in a qualitative change in type II topoisomerase that alters interaction of drug with the enzyme or enzyme-DNA complex. This is clearly supported by the findings with the somatic cell hybrid line, M,jr-7, which suggest that nearly complete recovery of sensitivity occurs in the presence of normal enzyme. While theoretically it is possible that resistance in this setting might be mediated by a decrease in total enzyme pools or functional activity, we believe our data effectively eliminate these as considerations in the VpmR@5 line. However, given that we have data only from crude extracts, it is quite conceivable that resistance could be mediated by the absence of a hypothetical “second factor” which might be required for drug-enzyme interaction.

In summary, our observations serve to further implicate type II topoisomerase as the major intracellular target for the antitumor effects of the epipodophyllotoxins. In addition, the data indicate that formation of the cleavable complex, rather than inhibition of normal catalytic activity, is the critical lesion responsible for cell death. In other studies, we have found that cross-resistance of the VpmR@5 line is confined to other agents which stimulate type II topoisomerase-mediated DNA cleavage, lending even more support to the concept of the enzyme as a multidrug target (21). It is quite likely that further study of the VpmR@5 line will define a previously undescribed mechanism of multidrug resistance with attendant biological and clinical implications.

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REFERENCES


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