DNA Strand Breaks Produced by Etoposide (VP-16,213) in Sensitive and Resistant Human Breast Tumor Cells: Implications for the Mechanism of Action

Birandra K. Sinha,1 Nissim Haim,2 Lata Dusre, Donna Kerrigan, and Yves Pommier

Clinical Pharmacology Branch, Clinical Oncology Program [B. K. S., N. H., L. D.], and Laboratory of Molecular Pharmacology [D. K., Y. P.], National Cancer Institute, NIH, Bethesda, Maryland 20892

ABSTRACT

Platetropic resistant human breast cancer cells (MCF-7), selected for resistance to Adriamycin, were used to study the production of DNA strand breaks by etoposide (VP-16) and its relationship to drug cytotoxicity. It was shown that the resistant MCF-7 cell line was cross-resistant to VP-16, and the degree of resistance was found to be 125–200-fold. Alkaline elution studies indicated that the parental cell line was very sensitive to VP-16 which caused extensive DNA strand breakage. In contrast, little DNA strand breakage was detected in the resistant MCF-7 cells, even at very high drug concentrations, indicating a good agreement between strand breaks and cytotoxicity. Further studies indicated that the nuclei isolated from the parental cell line were more resistant to VP-16-induced DNA strand breaks than the intact cells, while the opposite was found in the resistant cell line. In addition, the alkaline elution studies in isolated nuclei showed only a 2-fold reduction of VP-16-induced DNA breaks in nuclei from the resistant cells. In agreement with this result, it was found that nuclear extract from the resistant cells produced 2–3-fold less VP-16-induced DNA breaks than that from the sensitive cells in 32P-end-labeled SV40 DNA. VP-16 uptake and efflux studies indicated that there was a 2–3-fold decrease in net cellular accumulation of VP-16 in the resistant cells. Although the reduced uptake of VP-16 and decreased drug sensitivity of topoisomerase II appear to contribute to the mechanism of action and the development of resistance to VP-16, they do not completely explain the degree of resistance to VP-16 in this multidrug-resistant MCF-7 cell line indicating that other biochemical factors, such as activation of VP-16, are also involved in drug resistance and suggesting that the resistance is multifactorial.

INTRODUCTION

The epipodophyllotoxin derivative, etoposide, produces both single- and double-strand DNA breaks in mammalian cells and isolated cell nuclei (1–7). Earlier work of Loike and Horwitz (8) has shown that the presence of cellular components and a free hydroxyl group at C-4' in the E-ring was essential for the DNA damage to occur. Subsequently, it was shown in an in vitro system that VP-16-induced DNA cleavage is caused by the interaction of the drug with topoisomerase II, resulting in a covalent enzyme-DNA complex (9, 10). Therefore, it is believed that the intracellular target of VP-16 and the related compound VM-26 is nuclear DNA topo II and that the topo II-mediated DNA strand breaks are responsible for VP-16-dependent cell killing of human tumors (2, 3, 7). Studies reported from our laboratory have shown that VP-16 is metabolized either by cytochrome P-450 or by peroxidases (prostaglandin or horseradish) to dihydroxy- and o-quinone derivatives of VP-16 (11–15). Furthermore, such enzymatic activation of the drug also forms reactive intermediates that covalently bind to purified DNA and proteins (12, 13, 15). We have therefore postulated that the metabolism of VP-16 and formation of reactive species from VP-16 and subsequent binding of these species to cellular macromolecules may be important in VP-16-induced cell killing.

Studies in drug-resistant (or multidrug-resistant) cells with VP-16 have shown a reduced formation of protein-associated DNA strand breaks (16–19). This decrease in drug-induced DNA damage has been reported to arise from the modification of topo II activity in the resistant cells. We have recently reported that human breast tumor (MCF-7) cells made resistant to Adriamycin (ADR) also became resistant to VP-16 and had the multidrug-resistant phenotype (20). We have also shown that the development of resistance in the MCF-7 cells also induces changes in activation and detoxification enzymes, i.e., glutathione peroxidase and glutathione transferase, resulting in enhanced elimination of peroxides and free radicals (21–23).

In the present study, we have used both MCF-7-sensitive (WT) and -resistant cell (ADR) lines to study VP-16 uptake, VP-16-induced DNA strand breaks, and their relationship to cytotoxicity. Our findings show that VP-16 resistance was accompanied by reduced uptake of the drug and decreased VP-16-induced DNA breaking activity due to alterations in nuclear topo II activity. However, the decrease in the drug accumulation (2–3-fold), and alteration in topo II activity (2–3-fold in ADR cells do not completely explain the degree of resistance to VP-16 (125–200), indicating that other factors may also be involved in resistance to VP-16 in this multidrug-resistant MCF-7 cell line.

MATERIALS AND METHODS

VP-16,213 (NSC 1415140) was a gift from Bristol-Myers Co. (Syacuse, NY) and was dissolved in dimethyl sulfoxide immediately before experiments. Control cells were treated with equivalent concentrations of dimethyl sulfoxide. [3H]VP-16 labeled in the aromatic rings only (400 mCi/mmol; >99% pure) was obtained from Moravek Biochemicals, Inc., Brea, CA. [3H]Thymidine (56 mCi/mmol), and [3H]thymidine (80.9 mCi/mmol) were obtained from New England Nuclear, Boston, MA. SV40 and λ HindIII DNAs; Banl, EcoRI, HpaI restriction endonucleases; T4 polynucleotide kinase; and agarose were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Phosphatase was purchased from New England Biolabs (Beverly, MA) and [γ-32P]ATP from New England Nuclear Research Products (Boston, MA). XAR-5 films (Eastman Kodak Co., Rochester, NY) were used for autoradiography.

The human breast tumor (MCF-7) WT and ADR cells (kindly provided by Dr. Ken Cowan) were grown in monolayer in exponential growth phase in IMEM supplemented with 2 mM glutamine, gentamicin (100 units/ml; 10 ml/liter) and 5% fetal bovine serum (Grand Island Biological Co., Grand Island, NY) under an atmosphere containing 5% CO2. The selection of the resistant cells was achieved by treating WT cells with stepwise increasing concentrations of Adriamycin (Drug Development Branch, National Cancer Institute, NIH, Bethesda, MD) (20, 21).

VP-16-induced cytotoxicity was determined by either colony formation (drug treatment for 1 and 2 h) or growth inhibition assay (continuous exposure). The cells were trypsinized, plated (15,000 cells/ml
overnight and then dried on a No. 3MM paper with a lyophilizer. Dried markers and were run in the same gels. Gels were run at 2-3 v/cm before being loaded into 1% agarose gels in Tris-borate-EDTA buffer. 

The salt extraction was carried out by gentle rotation for 30 min. The nuclei were obtained by incubating the cells in 0.27% Triton X-100 for 10 min at 4°C. The nuclear pellets were then resuspended in the buffer. Nuclei were obtained by incubating the cells in 0.27% Triton X-100 for 10 min at 4°C. The nuclear pellets were then isolated by centrifugation. Drug treatments of isolated nuclei were for 30 min at 37°C. VP-16 was removed by diluting the nuclei suspensions (20-fold) in NB at 0°C. [3H]Thymidine internal standard LI210 cells in NB at 0°C were then mixed with [14C]labeled nuclei and alkaline elution was performed as described above.

Nuclei from the [14C]thymidine-labeled MCF-7 cells were prepared as described previously (16). Pelleted cells were suspended in NB at 4°C and the cells were washed with NB and resuspended in the buffer. Nuclei were obtained by incubating the cells in 0.27% Triton X-100 for 10 min at 4°C. The nuclear pellets were then isolated by centrifugation. Drug treatments of isolated nuclei were for 30 min at 37°C. VP-16 was removed by diluting the nuclei suspensions (20-fold) in NB at 0°C. [3H]Thymidine internal standard LI210 cells in NB at 0°C were then mixed with the [14C]-labeled nuclei and alkaline elution was performed as described above.

Nuclear extracts from the MCF-7 cells were prepared as described previously (27). All extractions were performed at 4. The nuclei pellets were suspended in NB containing 0.35 m NaCl (final concentration). The salt extraction was carried out by gentle rotation for 30 min. The nuclei were then spun down at 1800 rpm for 20 min. The supernatants were collected and recentrifuged for 10 min to remove any nuclei or insoluble materials.

DNA topo II was purified from leukemia (L1210) cell nuclei, as described previously (16). End-labeling of SV40 DNA was performed as follows: (a) native supercoiled SV40 DNA was first linearized by cutting at the BamHI restriction site; (b) the 5'-DNA termini were then dephosphorylated with calf alkaline phosphatase and labeled with [γ-32P]ATP in the presence of polynucleotide kinase; and (c) finally, a second cut was introduced with HpaII. This second cut being at approximately 50 base pairs from the labeling site, any fragment bigger than 50 base pairs could arise only from the longer fragment.

DNA cleavage reactions were carried out by reacting 32P-end labeled DNA with purified topo II or nuclear extracts in 0.01 M Tris-HCl (pH 7.5)-0.05 M KCl-5 mM MgCl2-0.1 mM EDTA containing 15 μg/ml bovine serum albumin for 30 min at 37°C. Reactions were performed in 40-μl volumes and stopped by adding sodium dodecyl sulfate (1% final concentration). Proteinase K (0.5 mg/ml) was then added and the reaction mixtures were incubated for an additional 30 min at 50°C. The DNA was extracted with phenol:chloroform (1:1). Loading buffer was added to each sample, which was then heated at 65°C for 2 min before being loaded into 1% agarose gels in Tris-borate-EDTA buffer. 32P-end labeled fragments of XHindIII EcoRI DNA were used as DNA markers and were run in the same gels. Gels were run at 2-3 v/cm overnight and then dried on a No. 3MM paper with a lyophilizer. Dried gels were autoradiographed with Kodak XAR-5 films (28).

RESULTS

The relative cytotoxic effects of VP-16 to WT and ADRR cells under different exposure conditions, e.g., 1 h and during continuous exposures, are shown in Fig. 1, and the relative 50% inhibitory concentration and the resistance index obtained under various conditions are summarized in Table 1. These data clearly show that the ADRR cell line is highly resistant to VP-16 and, that the VP-16-induced cytotoxicity increases with the time of drug exposure.

Since multidrug-resistant cells in general have transport defects (29, 30) which may explain the differential cytotoxic effects of the drug, we examined the cellular accumulation of VP-16 in these two cell lines. As shown in Fig. 24, the steady state concentration of VP-16 was significantly higher (2-3-fold) in the WT cells than in the ADRR cells. The uptake of VP-16 in both cell lines was rapid and linear up to 5 min.

The efflux of VP-16 from WT and ADRR cells with time was

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**Table 1 Relative cytotoxicity of VP-16 in sensitive (WT) and resistant (ADR) cells**

<table>
<thead>
<tr>
<th>MCF-7 cells</th>
<th>Exposure time (h)</th>
<th>IC50 WT (μM)</th>
<th>IC50 ADR</th>
<th>Resistance index</th>
</tr>
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<tr>
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<tr>
<td>1</td>
<td>5</td>
<td>600</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.5</td>
<td>600</td>
<td>170</td>
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<tr>
<td>Continuous</td>
<td>0.08</td>
<td>20</td>
<td>250</td>
<td></td>
</tr>
</tbody>
</table>

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IC50, VP-16 concentration required for 50% cell kill as determined by clonogenic assay (1 and 2 hr treatment) or 50% growth inhibition (continuous exposure).

IC50 ADR cells/IC50 WT cells.
rapid and most of the drug was released within 10–15 min. However, a small fraction (5–10%) of VP-16 remained associated with the cells even after 60 min and may represent an irreversibly bound fraction of the drug and/or metabolites. Cellular accumulation of VP-16 was found to be linear as a function of VP-16 concentrations, up to 100 μM, and at steady state, a 2–3-fold difference in drug accumulation was observed between the two cell lines at all drug concentrations examined (Fig. 2B).

Small differences (2–3-fold) in the net cellular accumulation of VP-16 in these two cell lines do not appear to account for the large difference (200-fold) in the cytotoxicity and resistance to VP-16. Since it is possible that cytotoxicity of VP-16 results from topo II-mediated DNA cleavage, we compared the production of DNA breaks by VP-16 in the WT and ADRR cells. As shown in Fig. 3, very few DNA strand breaks were produced in the ADRR cells, even at VP-16 concentrations up to 500 μM. In contrast, a significant amount of DNA breaks were produced by low concentrations of VP-16 in WT cells. DNA single-strand breaking frequency in rad equivalents as a function of drug concentration in the two cell lines is summarized in Fig. 3B.

Experiments were then carried out in isolated nuclei to investigate further the possibility that any drug transport or activation defect could be responsible for differential VP-16-induced DNA single-strand breaking activity in the intact cells. VP-16 treatment were performed for only 30 min because longer incubation resulted in the appearance of endogenous DNA breaks in the untreated nuclei which rendered the analysis of VP-induced breaks less clear. The data are presented in Fig. 3B. The difference between isolated nuclei from WT and ADRR cells was much smaller than that observed in the intact cells. It is noteworthy that VP-16 caused significantly less DNA breaks in isolated nuclei from WT cells than in the intact cells while more DNA breaks were detected in isolated nuclei from the ADRR cells than from the intact ADRR cells. A comparison of the data in intact cells and nuclei is presented in Table 2, which shows that VP-16 is 2–3-fold less active in isolated nuclei from ADRR cells than from isolated nuclei from WT cells. These results suggest that nuclear modifications in the resistant MCF-7 cells are partially responsible for VP-16 resistance.

In order to examine further whether the reduced formation of VP-16-induced DNA breaks in isolated nuclei from ADRR cells was due to altered topo II activity, nuclear extracts from both cell lines were compared. Increasing concentrations of nuclear extracts were then reacted with 32P-end-labeled SV40 DNA in the presence of 50 μM VP-16 (Fig. 4). The DNA-breaking activity of these nuclear extracts was compared to those of purified LI210 topo II and LI210 nuclear extracts. The DNA cleavage patterns induced by VP-16 in the presence of nuclear extracts from LI210, WT, and ADRR cells were similar and were comparable to that induced in the presence of purified LI210 topo II. LI210 nuclear extracts, however, were more potent than the WT nuclear extracts (Fig. 4). The ADRR nuclear extracts were less sensitive to VP-16 than WT nuclear extracts suggesting that ADRR cells had lower VP-16-sensitive topo II activity.

 Autoradiography films corresponding to Fig. 4 and additional experiments performed with 0.5-μg nuclear extracts (12.5 μg/
VP-16-INDUCED DNA DAMAGE IN HUMAN TUMOR CELLS

Fig. 4. Double strand breaks induced by VP-16 in the presence of WT and ADR® MCF-7 cell nuclear extracts in SV40 DNA. 32P-end-labeled SV40 DNA (control SV40 DNA) was reacted with purified LI210 topo II in the absence or presence of VP-16 (50 μM) ([topo II] and [topo II + VP-16] lanes, respectively). The indicated amounts of nuclear extract were reacted with DNA in the presence or absence of VP-16. Reactions were stopped after 30 min incubation at 37°C by adding 1% sodium dodecyl sulfate and 0.5 mg/ml proteinase K (final concentration). After an additional 30-min incubation at 50°C, the DNA was extracted with phenol-chloroform and loaded into a 1% agarose gel in Tris-borate EDTA buffer. The gel was dried and autoradiography was performed. 32P-end-labeled HindIII EcoRI restriction fragments of λ were used as DNA markers. The size of these markers in base pairs is: 564, 831, 983, 1584, 1904, 2027, 3530, 4277, and 4973 (bottom to top).

ml) were used to quantify topo II-mediated DNA cleavage produced in the presence of 50 μM VP-16 at several DNA cleavage sites and is summarized in Fig. 5. Topo II-mediated DNA cleavage was greater with LI210 nuclear extract than with nuclear extract from WT and ADR® cells. WT nuclear extract was approximately twice more potent than the ADR® extract. These results are in good agreement with those obtained with isolated nuclei (Fig. 4B) and indicate a 2-fold resistance of topo II from ADR® cells to VP-16.

DISCUSSION

Several investigators have reported that topo II-catalyzed DNA strand breaks are responsible for the antitumor activity of VP-16 (2, 3, 5–7). Cellular resistance to VP-16 and antitumor DNA intercalators, is accompanied by decreased drug-induced topo II-mediated DNA-cleaving activity in both intact cells and isolated nuclei (17–19). The results presented here show that MCF-7 cells selected for resistance to Adriamycin are also significantly resistant to VP-16. In the resistant cell, there is a marked decrease in VP-16-induced DNA single strand breaks and this decrease (in single strand breaks) (100–200-fold) is in good agreement with the degree of resistance observed for VP-16 in this cell line. Furthermore, there is a good correlation with the topo II-mediated DNA cleavage and VP-16-induced cytotoxicity. The decrease in DNA strand breaks was also observed in isolated nuclei of the ADR® cells compared to WT cells. The decreased formation of VP-16-induced DNA single strand breaks observed in the ADR® cells appears to be related partially (2–3-fold) to a decrease in the nuclear activity of topo II in the ADR® cells. Topo II modifications have also been documented with topo II-active drugs in other resistant cell lines (17–19, 28, 31). However, our observation in MCF-7/ADR® cells is unique because this cell line has been characterized as typical pleotropic cells with overexpression of the Mf, 170,000 protein.

Resistance to VP-16 in MCF-7 cells was also accompanied by a 2–3-fold decrease in the net cellular accumulation of VP-16 in the ADR® cells. Thus, the development of VP-16 resistance in the multidrug-resistant MCF-7 cells is, in part, due to defects in the transport of VP-16 in the resistant cells and alteration in the topo II activity resulting in decreased formation of DNA strand breaks. These two observations, i.e., a 2–3-fold decrease in cellular accumulation for VP-16 and 2–3-fold decrease in VP-16-induced topo II-mediated DNA cleavage activity, taken together, however, are not sufficient to explain 125–200-fold resistance in MCF-7 cells. Thus, it appears that other biochemical mechanisms contribute to the development of resistance to VP-16 ADR® cells. Furthermore, the findings that the nuclei prepared from WT cells formed significantly less VP-16-induced DNA breaks than the intact cells would suggest that topo II activity (including its activation) was lost selectively during the isolation of nuclei from WT cells. Similar observations have been described previously in other cell lines (7, 26). It is also possible that the intracellular distribution of VP-16 is different in the resistance cells or that some other factors in the cytosol of the WT cells were necessary for the VP-16-induced DNA strand-breaking activity.

The biochemical nature of the “necessary factors” is under study at the present time. It is possible to postulate that VP-16 is enzymatically activated by cytoplasmic enzymes to a reactive o-quinone derivative or a quinone methide which binds irreversibly to proteins and DNA (12–13, 15, 32). This activation
of the drug has been shown to be catalyzed by cytochrome P-450. Of interest is our previous finding that the ADR cells are rapidly detoxified in the ADRR cells since ADRR cells show a significant increase in both glutathione peroxidase and glutathione transferase activities (20, 21). The function of these two enzymes is to detoxify organic free radicals and reactive alkylating species. The fact that ADR cells are also resistant to benzo(a)pyrene (20) is consistent with the activation/detoxification hypothesis.

In conclusion, the association of decreased drug uptake, decreased formation of protein associated DNA strand breaks in the resistant cell line may have broad implications in the phenomenon of multidrug resistance.

REFERENCES

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