Expression of Topoisomerase II, Bcl-2, and p53 in Three Human Brain Tumor Cell Lines and Their Possible Relationship to Intrinsic Resistance to Etoposide

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ABSTRACT
We characterized three human brain tumor cell lines (D54, HBT-20, and HBT-28) with respect to resistance to etoposide (VP-16), a topoisomerase II-reactive drug. All three cell lines were inherently resistant to VP-16 when compared to other human cell lines, with D54 showing the greatest resistance using colony formation assays. Resistance to VP-16 has been attributed to decreased drug uptake and changes in topoisomerase II; however, drug uptake and topoisomerase II protein levels (immunoblot) were no lower in D54 than in HBT-20 and HBT-28, cell lines relatively more sensitive to VP-16. More to the point, measurement of topoisomerase II-mediated DNA cleavage of cellular DNA after treatment with VP-16 showed that the topoisomerase II in these cells was active. These data indicate mechanisms other than those attributable to decreased drug uptake or altered topoisomerase II exist for clinical resistance to VP-16. VP-16-induced DNA cleavage has been associated with apoptosis in some cell lines; however, neither DNA laddering nor morphological changes characteristic of apoptosis were detected in these cell lines after treatment with VP-16. Bcl-2 and mutant p53 were present in these cells. Either of these conditions can prevent apoptosis and could explain a dissociation between the proximal mediator of VP-16-induced cytotoxicity (topoisomerase II-DNA complex formation) and cellular death.

INTRODUCTION
Topo II is an enzyme whose actions to alter DNA topology are required for cell division. Several chemotherapeutic agents, including VP-16, are believed to exert their cytotoxicity via stabilization of a topo II-DNA complex with inhibition of the religation of DNA strand breaks produced by the enzyme itself. The mechanisms by which complex stabilization and DNA strand breaks lead to cell death have not been elucidated; but, in general, the magnitude of drug-induced DNA strand break production correlates with the magnitude of drug-induced cytotoxicity (1, 2). Resistance to topo II-reactive drugs has been shown to occur as a result of decreased intracellular accumulation of drug (3), decreased expression of the topo II gene (4), or expression of an altered, drug-resistant form of the enzyme (4).

Although the frequency of drug-induced, topo II-mediated DNA strand breaks has generally been shown to correlate with the degree of cytotoxicity, there are exceptions (5). These breaks are reversible upon removal of the drug, yet cell death still occurs (5). Thus, complex formation and DNA strand breakage appear to be necessary but in themselves not sufficient for cytotoxicity to occur. There is evidence that these DNA strand breaks trigger apoptosis or programmed cell death (6, 7). If apoptosis is the ultimate cause of cytotoxicity in response to treatment with topo II-reactive drugs, then aberrations in the apoptotic pathway could also result in drug resistance. Numerous genes have been shown to influence the occurrence of apoptosis: an increase in apoptosis has been seen with expression of wtp53 (8, 9), c-myc (10), and interleukin 1B-converting enzyme (11), while inhibition of apoptosis has been seen with expression of Bcl-2 (12, 13).

We report here the characterization of VP-16 resistance in three human brain tumor cell lines. Initial efforts focused on measuring drug uptake and evaluating topo II levels, since these are the mechanisms of VP-16 resistance which have been previously described (14, 15). Surprisingly, we found that VP-16 was taken up by these cells and a drug-sensitive topo II protein was present in all three cell lines. Treatment with doses of VP-16, which resulted in DNA strand breaks and inhibition of colony formation, failed to induce apoptosis. Preliminary studies show that both Bcl-2 and mutant p53 are present in these cells. Either of these conditions could block apoptosis and account for a dissociation between the proximal mediator of VP-16-induced cytotoxicity (topoisomerase II-DNA complex formation) and cellular death. Additional studies are under way to determine whether p53 or Bcl-2 play a role in the VP-16 resistance of these cells.

MATERIALS AND METHODS
Chemicals and Reagents. VP-16 was a gift from Drs. Byron Long and James H. Keller (Bristol-Meyers Co). Amscarine was obtained from the National Cancer Institute (Bethesda, MD). [14C]thymidine (59 mCi/mmol), [methyl-3H]thymidine (20 mCi/mmol), and [3H]H2O (1 mCi/ml) were obtained from New England Nuclear (Boston, MA). 125I-labeled protein A and [α-32P]ATP were obtained from Amersham (Arlington Heights, Ill.).

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3 The abbreviations used are: topo II, topoisomerase II; VP-16, etoposide; kDNA, kinetoplast DNA; FSP-50, 50% strand passage; wtp53, wild-type p53.
Topo II, Bcl-2, and p53 in VP-16-resistant Cells

were performed using standard techniques (18). Specific RNA values were used to determine the molar amount of topo II protein levels as previously described by Kaufmann et al. (16). The antihuman topo II polyclonal antibody was used at a 1:500 dilution. The antibody precipitated without the use of proteinase K at an elution rate of 2 ml/h for 15 h.

**Nuclear Extracts.** Cells were washed with isotonic buffer (150 mM NaCl, 5 mM MgCl\textsubscript{2}, 2 mM KH\textsubscript{2}PO\textsubscript{4}, 1 mM EGTA, 10% glycerol, 0.1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride), then lysed by incubation for 10 min in isotonic buffer plus 0.3% Triton X-100. Isolated nuclei were then extracted for 30 min in isotonic buffer, with the final NaCl concentration increased to 350 mM. The nuclei were then centrifuged at 100,000 \times g (23). Protein in the supernatant was quantified using the Bio-Rad (Richmond, CA) method.

**Decatenation.** kDNA was labeled with [methyl-\textsuperscript{3}H]thymidine, then isolated from Crithidia fasciculata. Topo II catalytic activity in nuclear extracts was quantified as previously described (1). Topo II-mediated DNA strand passage resulted in decatenation of the intertwined kDNA network. After separation on a 1% agarose gel, the catenated kDNA network remaining in the well and the decatennated minicircles and maxicircles that enter the gel were excised separately and counted. Data are expressed as the fraction of strand passage, calculated as follows:

\[
\text{FSP} = \frac{(\text{fraction of kDNA in well})_{\text{no protein}} - (\text{fraction of kDNA in well})_{\text{with protein}}}{(\text{fraction of kDNA in well})_{\text{no protein}}}
\]

**Drug-induced DNA-Protein Cross-Linking.** SDS/KCl precipitation of \textsuperscript{32}P 3' end-labeled SV40 DNA was quantified using a modification of the method described by Liu et al. (24). The reaction (50 \mu l) was carried out in 10 mM Tris, 10 mM KCl, 50 mM NaCl, 5 mM MgCl\textsubscript{2}, 1 mM EDTA, 15 \mu g/ml BSA, and 1 mM ATP for 30 min at 37°C. The amount of nuclear extract used in each assay was 1.25 times the amount needed for FSP-50. Reactions were stopped by adding two volumes of 2% SDS, 2 mM EDTA, 0.5 mg/ml salmon sperm DNA, and 0.2 nM NaOH. Protein-DNA complexes were then precipitated by increasing the KCl concentration to 190 mM. Results are expressed as the cpm precipitated in the presence of drug minus the cpm precipitated without drug.

**Internucleosomal DNA Fragmentation.** Approximately 10\textsuperscript{7} cells were lysed by incubation for 20 min on ice in 25 mM Tris (pH 8.0), 20 mM EDTA, 10 mM EGTA, and 0.5% Triton X-100. After phenol-chloroform extraction, the DNA was isolated by ethanol precipitation. The DNA was treated for 30 min at 37°C with 10 \mu g/ml RNase A, then analyzed on a 1.4% agarose gel.

**RESULTS**

**Colony Formation Assay.** The sensitivity of these three cell lines to VP-16 was evaluated using the colony formation assay (Fig. 1 and Table 1). Although these cells had not been selected in vitro for drug resistance, all three cell lines were relatively resistant to VP-16 when compared with resistance in several other human tumor cell lines in which the IC\textsubscript{50} was <5 \mu M VP-16 with a 1-h exposure (1, 14, 15, 25).
HBT-20, which had 50% higher protein levels and slightly more four times more resistant to VP-16 than was HBT-28 at the and a 2-fold higher concentration of VP-16 in D54, D54 was almost identical bevels of topo II protein in D54 and HBT-28 topo II were evaluated using immunoblotting (Table 1). Despite this assay measures drug both in and on the cell, we in D54 cells thus cannot be explained by reduced drug accumu-

reported). We also measured the amount of drug associated with pellets. 3H counts were then used to determine VP-16 and H2O associ-
in D54 than in the other two lines. The resistance to VP-16 seen in D54 cells thus cannot be explained by reduced drug accumu-

Effect of VP-16 on colony-forming ability. Cells were treated for 1 h with VP-16, then plated and allowed to grow 12 days prior to counting colonies. Linear regression lines were determined using duplicate points for each drug concentration. R value of linear regression: D54, 0.85; HBT-20, 0.97; and HBT-28, 0.91.

Table 1  Summary of findings in human brain tumor cell lines

<table>
<thead>
<tr>
<th></th>
<th>IC_{50}°</th>
<th>[VP-16]°</th>
<th>Topo II</th>
<th>FSP-50°</th>
<th>p53°</th>
<th>Bcl-2°</th>
</tr>
</thead>
<tbody>
<tr>
<td>D54</td>
<td>92</td>
<td>2.1 ± 0.62</td>
<td>100</td>
<td>0.22 ± 0.01</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>HBT-20</td>
<td>61</td>
<td>1.5 ± 0.05</td>
<td>146 ± 62</td>
<td>0.15 ± 0.01</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>HBT-28</td>
<td>22</td>
<td>1.1 ± 0.19</td>
<td>91 ± 6</td>
<td>0.16 ± 0.01</td>
<td>+++</td>
<td>+++</td>
</tr>
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° IC_{50} determined by colony formation assay after treatment with VP-16 for 1 h.

° Cells were incubated with either [3H]VP-16 or [3H]H2O for 1 h, then centrifuged through Versilube F50 silicone fluid to obtain cell pellets. 3H counts were then used to determine VP-16 and H2O associated with the cell pellets and to calculate the VP-16 concentration. Results are from triplicate experiments with SEs given.

° Cellular proteins were isolated and assayed for topo II levels by immunoblotting. Quantitative values were determined using Phosphoimage scanning of autoradiographies from three separate experiments. D54 was arbitrarily assigned a value of 100. Results are from triplicate experiments with SEs given.

° The amount of protein needed for strand passage (decatenation) of 50% of the kDNA. Results are from triplicate experiments with SEs given.

° p53 and Bcl-2 protein levels in whole cell lysates were detected by immunoblot analysis using enhanced chemiluminescence detection.

Evaluation of Cellular VP-16 Concentration. Resistance to VP-16 can result from reduced intracellular drug levels, as would occur in cell lines with overexpression of mdr-1 (3). We assayed for expression of the mdr-1 gene using Northern blot and found no expression in these three cell lines (data not shown). We also measured the amount of drug associated with these three cell lines (Table 1) and found higher concentrations in D54 than in the other two lines. The resistance to VP-16 seen in D54 cells thus cannot be explained by reduced drug accumulation. Since this assay measures drug both in and on the cell, we cannot rule out altered cellular distribution of VP-16 as the cause of resistance.

Evaluation of Topo II Levels. Total cellular levels of topo II were evaluated using immunoblotting (Table 1). Despite almost identical levels of topo II protein in D54 and HBT-28 and a 2-fold higher concentration of VP-16 in D54, D54 was four times more resistant to VP-16 than was HBT-28 at the IC_{50}.

Evaluation of Apoptosis. The resistance to VP-16 despite the presence of drug-induced, topo II-mediated DNA

Drug accumulation compared with HBT-28, was 3-fold more resistant to VP-16 at the IC_{50}.

Evaluation of Topo II Function. The resistance to VP-16 despite the presence of seemingly adequate quantities of topo II protein and uptake of VP-16 suggested that abnormally low topo II activity might explain the VP-16 resistance in these cells. To indirectly evaluate topo II activity in intact cells, assays were performed using alkaline elution to quantify VP-16-induced, topo-II-mediated DNA cleavage (Fig. 2). The frequency of single-strand breaks was determined both with and without proteinase K treatment prior to elution. The number of single-strand breaks detected in the absence of proteinase K was <5% of the number of breaks seen in the presence of proteinase K (data not shown), indicating that the majority of the breaks were accompanied by DNA-protein cross-linking. Only minor differences were noted among these cell lines, with substantial DNA cleavage at 2–20 μM concentrations of VP-16. Despite the presence of similar numbers of breaks in all three cell lines at these drug concentrations, D54 and HBT-20 cells were more resistant to VP-16-induced cytotoxicity than were HBT-28 cells.

Direct analysis of the catalytic activity of topo II in nuclear extracts (350 mm NaCl) was measured by decatenation of kDNA. Prior to decatenation, the intertwined kDNA network is unable to enter a 1% agarose gel. Topo II activity results in the formation of minicircles and maxicircles that are capable of entering the gel. The decatenating activity of a given amount of nuclear extract protein was almost identical in all three cell lines, with the amounts that resulted in FSP-50 in D54, HBT-20, and HBT-28 being 0.22 μg, 0.15 μg, and 0.16 μg, respectively.

Using amounts of nuclear extracts with identical decatenating activities, quantitation of drug-induced, topo II-mediated DNA cleavage was performed using the SDS/KCl precipitation of 32P 3' end-labeled SV40 DNA. Topo II-DNA complexes are stabilized by addition of drug, and SDS/KCl results in covalent linkage of topo II to the 5' end of radiolabeled DNA and in precipitation of this linked complex. Both VP-16 and amsacrine induced higher levels of DNA-protein cross-linking using nuclear extracts from D54 and HBT-20 than those levels induced using extracts from HBT-28 (Fig. 3).

Evaluation of Apoptosis. The resistance to VP-16 despite the presence of drug-induced, topo II-mediated DNA
Fig. 2 VP-16-induced single-strand break frequency. 14C-labeled cells were treated with VP-16 for 1 h at 37°C, then the number of breaks was quantified by alkaline elution with proteinase K at pH 12.1. 3H-labeled L1210 leukemia cells were used as an internal control. The frequency of strand breaks is expressed in rad-equivalents, or the amount of radiation required to produce a comparable number of breaks. Each point is the mean ± SE of at least three independent experiments. Where less than three experiments were performed, individual points are shown.

Fig. 3 SDS/KCl precipitation of 32P 3' end-labeled SV40 DNA using nuclear extracts in the presence of VP-16 or 4'-(9-acridinylamino) methanesulfon-m-anisidine (mAMSA). Nuclear extracts containing amounts of topo II equal to 1.25 times the FSP-50 (D54, 0.33 µg; HBT-20, 0.22 µg; HBT-28, 0.24 µg) were incubated with labeled SV40 DNA in the absence or presence of drug for 30 min at 37°C. Data are expressed as the counts precipitated in the presence of drug minus the counts precipitated in the absence of drug.

strand breaks indicated that resistance to VP-16 may be mediated by changes in the cytotoxicity pathway distal to the formation of DNA strand breaks. Since apoptosis has been implicated as the ultimate mediator of cell death caused by topo II-reactive drugs (6, 7), the three cell lines were evaluated for the presence of VP-16-induced apoptosis. Cells were treated for 1 h with concentrations of VP-16 equal to the IC50, as measured by colony formation assays. Evaluation 8 and 72 h after a 1-h treatment with VP-16 revealed no internucleosomal DNA degradation characteristic of apoptosis in these brain tumor cell lines, although degradation was detected in the HL-60 human leukemia cells used as a positive control (Fig. 4). Cells were also stained with acridine orange and evaluated at 8, 48, and 72 h after a 1-h treatment with VP-16 (data not shown). There was no increase in nuclear condensation as is typical with apoptosis. Although cellular blebbing was seen, this was associated with an
whether mutant p53 was present in these cells, immunoprecipita-
tion assays were preformed with Ab240, a mutant-specific
antibody (27). p53 was immunoprecipitated by Ab240 in each of
these cell lines, indicating that each cell line contains mutated
p53 in the D54 cell line, but no change in p53 in HBT-20 and
HBT-28 respectively.

Evaluation of p53 and Bcl-2. Introduction of wtp53 into
cells has been shown to increase apoptosis (8, 26), whereas
introduction of Bcl-2 inhibits apoptosis (12, 13). The cell lines
were therefore evaluated to determine whether the presence of
Bcl-2 or the absence of wtp53 could be acting to inhibit apop-
tosis that would otherwise be triggered by topo II-mediated
DNA strand breaks. Bcl-2 was detected in all three cell lines;
however, D54 had much lower levels than either HBT-20 or
HBT-28 (the levels detected in these two cell lines are compar-
able to levels we detect in HL-60 cells), and those levels did not
change after treatment with VP-16 (Fig. 5A). Likewise, using
anti-p53 (Ab1801), p53 was detected in all three cell lines, with
D54 again having much lower levels than the other two cell
lines. Treatment with VP-16 resulted in a marked increase of
p53 in the D54 cell line, but no change in p53 in HBT-20 and
only a minor increase in HBT-28 (Fig. 5B). To determine
whether mutant p53 was present in these cells, immunoprecipita-
tion assays were preformed with Ab240, a mutant-specific
antibody (27). p53 was immunoprecipitated by Ab240 in each of
these cell lines, indicating that each cell line contains mutated
p53 (data not shown).

increase rather than decrease in cell size, suggesting necrosis
rather than apoptosis.

DISCUSSION

Resistance to VP-16 in other cell lines has been attributed
to decreased intracellular drug levels, decreased levels of topo II
enzyme, or the presence of an altered topo II enzyme with
decreased ability to form topo II-DNA complexes in the pres-
ence of drugs like VP-16 (3, 4). The levels of cell-associated
VP-16 are lower than the 3.7 μM seen in VP-16 sensitive HL-60
cells (28). However, this does not adequately explain the resis-
tance, since D54, the cell line with the highest amount of
cell-associated VP-16, is the least sensitive to VP-16. Besides,
DNA strand breaks occur in all three brain tumor cell lines,indicating the relatively uniform penetration of drug into the cell
nucleus. Likewise, differences in topo II protein levels do not
adequately explain the resistance to VP-16, since D54 and
HBT-28 have similar protein levels but D54 is 4-fold more
resistant despite having higher cellular VP-16 levels. Evaluation
of VP-16-induced DNA strand breaks by alkaline elution re-
vealed comparable topo II cleavage activity in all three cell
lines, suggesting that pools of topo II with varying abilities to
participate in DNA damage and to cause cytotoxicity may exist.
However, evaluation of topo II in nuclear extracts demonstrated
the similarity of topo II catalytic and DNA cleavage activity in
all three cell lines.

Although these cell lines were not selected in vitro for
resistance to VP-16, all three showed some degree of resistance
to VP-16 compared with other, well-characterized cell lines.
The degree of inhibition of colony formation after a 1-h treat-
ment with VP-16 was less than that seen in HL-60/AMSA, a
drug-resistant HL-60 cell line (IC50 = 10 μM; Ref. 1). Inhibition
of colony formation was comparable to that seen in solid tumor
cell lines selected in vitro for resistance [HT1080 versus
HT1080/DR4, IC50 = <5 μM versus >200 μM (25); Chinese
hamster ovary versus VpR8, IC50 = 5 μM versus 60 μM (14);
MCF7 versus ADRR, IC50 = 5 μM versus 600 μM (15)]. Despite
this resistance, all three cell lines had DNA strand breaks of at
least 1000 rad-equivalents after treatment with VP-16 concen-
trations as low as 10 μM. This extent of DNA breakage results
in cytotoxicity in a number of other cell systems (15, 25).
However, the number of breaks does not always correlate with
cytotoxicity, and the exact mechanism by which drug-induced
topo II-DNA strand breaks result in cell death has not been
elucidated. The lack of correlation between VP-16-induced,
topo II-mediated DNA strand breaks and cytotoxicity suggests

Fig. 4 Internucleosomal DNA fragmentation induced by VP-16. DNA
was isolated from approximately 107 cells and analyzed on a 1.4%
agarose gel. Cells were either untreated or treated for 1 h with VP-16 at
concentrations equal to the IC50, by colony formation assay, then incu-
bated an additional 8 or 72 h prior to the isolation of DNA. HL-60 cells,
used as a positive control, were either untreated or treated for 4 h with
5 μM VP-16. Marker DNA is φX174 cut with Hae III.

Fig. 5 Immunoblot analysis of p53 and Bcl-2 expression. Each lane
contains 20 μg of total protein from lysates of untreated cells or cells
-treated for 1 h or 4 h with VP-16 at concentrations equal to the IC50,
by colony formation assay.
that resistance to VP-16 is mediated at a point in the cytotoxicity pathway distal to DNA breakage.

The ultimate mechanism by which topo II-reactive drugs cause cell death is unknown; however, apoptosis has been implicated (6, 7). In response to treatment with VP-16, no evidence of internucleosomal DNA degradation characteristic of apoptosis was found in these cells, nor were the morphological changes of apoptosis detected. The ability of cells to undergo apoptosis is an intrinsic property that may be cell-type specific. The lack of an intrinsic ability to undergo apoptosis may be part of the normal phenotype of some cell types and of tumors arising from these cells. On the other hand, the malignant nature of these cells may be a direct result of the inhibition of apoptosis resulting from an aberration in the signaling pathway between DNA damage and cell death. Both p53 and Bcl-2 have been shown to affect the occurrence of apoptosis. An increase in wtp53 expression occurs with radiation-induced apoptosis (8, 29). Likewise, introduction of wt p53 into cells with absent or mutated p53 has been shown to potentiate chemotherapy and radiation-induced apoptosis (8, 26). However, not all apoptosis requires p53 (8, 9). On the other hand, introduction of Bcl-2 can decrease apoptosis after withdrawal of growth factors in lymphoid (12) and neuronal (13, 30) cell lines. Bcl-2 also inhibits apoptosis induced by DNA damaging agents and specifically can inhibit apoptosis and cytotoxicity triggered by VP-16 without decreasing DNA strand breaks or DNA-protein cross-linking (31).

The presence of Bcl-2 in all three cell lines, and especially the higher levels in HBT-20 and HBT-28, suggest that Bcl-2 may inhibit progression along the apoptotic pathway that would otherwise be triggered by the occurrence of DNA strand breaks. Similarly, mutation of p53 in all three of these cell lines could prevent apoptosis. We cannot rule out the presence of wt p53, as well as mutant p53, but since p53 mutations are frequently dominant the presence of wtp53 would not preclude that mutant p53 is preventing apoptosis.

Our data show that, in contrast to what is seen in other cells, these cells do not undergo apoptosis in response to VP-16-induced, topo II-mediated DNA damage. Since these cells were not selected for resistance to VP-16, the mechanism of resistance present in these cells may more accurately reflect what is encountered in a clinical setting. Our current efforts are aimed at manipulation of the apoptotic pathway as a means of increasing sensitivity to VP-16.

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