

DNA Damage as a Basis for 4'-Demethylepipodophyllotoxin-9-(4,6-O-ethylidene- β -D-glucopyranoside) (Etoposide) Cytotoxicity¹

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ABSTRACT

The precise mechanism of action of 4'-demethylepipodophyllotoxin-9-(4,6-O-ethylidene- β -D-glucopyranoside) (VP-16), an important chemotherapeutic agent, has yet to be determined. VP-16 has been shown to cause single-strand breaks (SSBs) in DNA, but their relationship to cytotoxicity has not been determined. We have investigated the action of VP-16 using mouse leukemia L1210 cells in culture. By using the alkaline elution technique, we reaffirmed the occurrence of SSBs in DNA over the drug concentration range 1 to 60 μ M. We were able to demonstrate additional types of DNA damage in the form of DNA double-strand breaks and DNA-protein cross-links within the same dose range. The number of double-strand breaks formed per SSB was consistent over this dose range and greater than that found after exposure of L1210 cells to γ -irradiation. DNA SSBs and double-strand breaks were also shown to occur in isolated nuclei, indicating that cytoplasmic components are not required for this drug action. Colony formation by L1210 cells in soft agar decreased over a drug concentration range similar to that which produced DNA damage. The correlation between the effective dose range in the colony-forming assay and the DNA scission experiments supports the hypothesis that DNA breakage is responsible for drug cytotoxicity. The demonstration of strand scission in isolated nuclei may provide an experimental model for elucidating the exact mechanism of action of VP-16.

INTRODUCTION

VP-16⁴ is a semisynthetic derivative of podophyllotoxin. The structures of VP-16 and its parent compound are shown in Chart 1. Podophyllotoxin is known to bind to the microtubule subunit tubulin, inhibiting its polymerization into microtubules and arresting cell division in mitosis (13). VP-16, on the other hand, causes an increase in the proportion of cells with DNA content corresponding to S and G₂ phases of the cell cycle (4) and does not inhibit microtubule assembly at cytotoxic concentrations (8). Thus, it is unlikely that this drug acts by a mechanism similar to podophyllotoxin.

In 1976, Loike and Horwitz (9) studied the effect of VP-16 on the molecular weight of DNA in HeLa cells. Using alkaline sucrose gradient centrifugation, they determined that high-

molecular-weight DNA was converted to low-molecular-weight DNA after a brief exposure of cells to concentrations of VP-16 as low as 1 μ M. Upon drug removal, there was a reversion of low- to high-molecular-weight DNA. This suggested that the drug caused SSBs, which were rapidly repaired after drug removal. While these results are consistent with a mechanism of drug action based on DNA damage, they made no attempt to correlate DNA effects with cytotoxicity in HeLa cells. It is of interest that Loike and Horwitz observed no alteration in the sedimentation properties of purified HeLa DNA or adenovirus DNA following incubation with VP-16. Thus, strand breakage was not a direct effect of drug on DNA.

Based on the above data, we conclude that VP-16 does not exert its antitumor effect by inhibiting microtubule assembly. The alternative hypothesis, *i.e.*, a mechanism based on DNA damage, is attractive because it accommodates much of the existing data and because DNA is a target structure for many antitumor agents. In this paper, we attempted to test the DNA damage hypothesis by correlating such damage with cytotoxicity in an experimental tumor cell line. DNA damage was assayed by the alkaline elution technique (5). In addition to confirming the data of Loike and Horwitz (9), we have extended their findings by examining other forms of VP-16-induced DNA damage including DNA DSB and DNA-protein cross-links. We have also correlated the drug dose range that produces DNA damage with that which causes cytotoxicity. The effects of VP-16 on DNA in isolated nuclei were ascertained in order to determine if cytoplasm is required for drug activity and to provide an experimental model for future studies.

MATERIALS AND METHODS

Mouse leukemia L1210 cells, grown in suspension in Roswell Park Memorial Institute Medium 1630 containing 20% fetal calf serum, penicillin, and streptomycin, were used in all experiments. The doubling time was approximately 12 hr. Cells were labeled with [2-¹⁴C]thymidine (53 mCi/mmol; 0.01 μ Ci/mmol; New England Nuclear, Boston, Mass.) or with [*methyl*-³H]thymidine (20 Ci/mmol; 0.1 μ Ci/ml diluted with unlabeled thymidine to give a concentration of 10⁻⁶ M in the culture medium; New England Nuclear) approximately 20 hr before being used for experimentation.

VP-16 was a gift from Bristol Laboratories (Syracuse, N. Y.). It was dissolved in dimethyl sulfoxide, and unless otherwise specified most drug treatments were for 1 hr at 37°.

Cells were radiated on ice using a ¹³⁷Cs source (Mark I Irradiator; J. L. Sheppard and Associates, Glendale, Calif.) and kept cold until the time of elution. The rate of exposure was 2250 R/min.

The alkaline elution technique for assaying high-frequency DNA SSBs is a modification of one described in detail elsewhere (15). Cells containing ¹⁴C-labeled DNA were layered onto a polyvinyl chloride filter (pore size 2 μ m; Millipore Corp., Bedford, Mass.) and lysed with a solution of 2% sodium dodecyl sulfate, 20 mM disodium EDTA, and proteinase K, 0.5 mg/ml (Merck, Darmstadt, Germany). The DNA was

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⁴ The abbreviations used are: VP-16, 4'-demethylepipodophyllotoxin-9-(4,6-O-ethylidene- β -D-glucopyranoside), (etoposide, VP-16-213); SSB, single-strand break; DSB, double-strand break.

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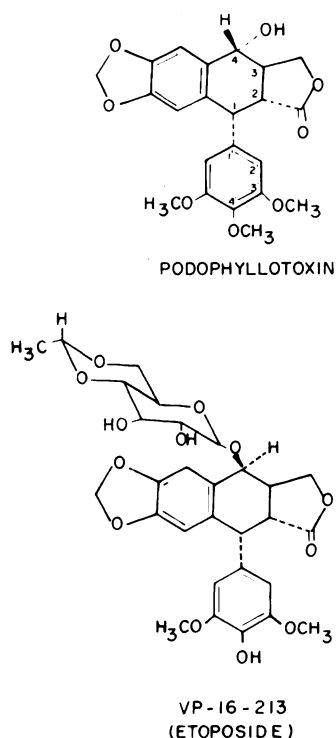


Chart 1. Molecular structures of podophyllotoxin and its derivative VP-16 (etoposide).

then eluted from the filter with tetrapropylammonium hydroxide (RSA Corp., Ardsdale, N. Y.), pH 12.1. Cells which contain [^3H]DNA and had received 2000 R prior to elution were included on each filter as internal standards. The elution flow rate was 0.16 to 0.2 ml/min, with a fraction interval of 5 min and a total elution time of 30 min. Results obtained using this assay were essentially identical to those obtained using the method reported previously by Zwelling *et al.* (15). DNA-protein cross-links were demonstrated by lysing cells with a lysis solution not containing proteinase K and eluting at 0.03 to 0.04 ml/min for 15 hr.

DNA DSBs were assayed by depositing 2.5×10^5 cells on a polycarbonate filter (2- μm pore size; Bio-Rad Laboratories, Richmond, Calif.) and lysing them as in the SSB assay. The native DNA was then eluted from the filter with 0.2% sodium dodecyl sulfate in a buffer of tetrapropylammonium hydroxide at pH 9.6. The elution flow rate was 0.04 ml/min with a fraction interval of 3 hr and a total elution time of 15 hr.

Isolated nuclei⁵ were prepared by first washing ^3H -labeled whole cells in cold Buffer A (1 mM KH_2PO_4 :5 mM MgCl_2 :150 mM NaCl :1 mM EGTA) at pH 6.4. The cells were resuspended in 1 ml Buffer A and were lysed with 9 ml of Buffer B (Buffer A plus 0.3% Triton X-100; Eastman Kodak Co., Rochester, N. Y.). Immediately, 40 ml more of Buffer A were added, and the nuclei were sedimented by centrifugation at 1000 rpm for 10 min. The presence of only isolated nuclei was confirmed by phase microscopy. Following resuspension in Buffer A, nuclei were treated with drug for 1 hr at 37° at a density of 10^6 nuclei/ml. The alkaline elution assay for both DNA SSBs and DSBs was then performed as usual except that internal standards were not used in these assays.

Cytotoxicity was measured by using a modification of the soft agar colony-forming assay of Chu and Fisher (1).

RESULTS

VP-16 produced both DNA SSBs and DNA DSBs in whole cells. The results of the DNA SSB assay in whole cells is shown

⁵ This technique was kindly communicated by Dr. Janek Filipski prior to publication.

in Chart 2. There was a dose-dependent increase in the elution rate of DNA from cells treated at 37° with VP-16 over a range of 5 to 20 μM . No DNA damage occurred in the cells treated at

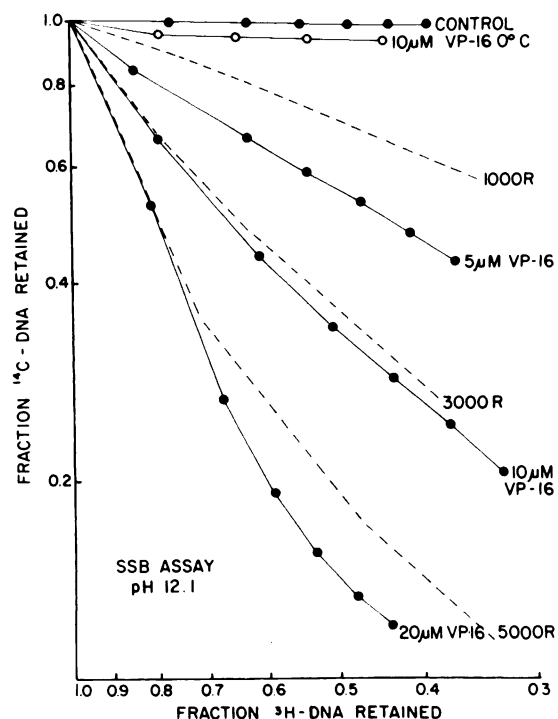


Chart 2. DNA SSBs in L1210 cells treated with VP-16 or γ -radiation. The fraction of retained ^{14}C -labeled DNA from experimental cells is plotted against the fraction of retained ^3H -labeled internal-standard DNA. The internal-standard cells were irradiated with 2000 R. The DNA was eluted at pH 12.1. Cells treated at 0° showed no SSBs.

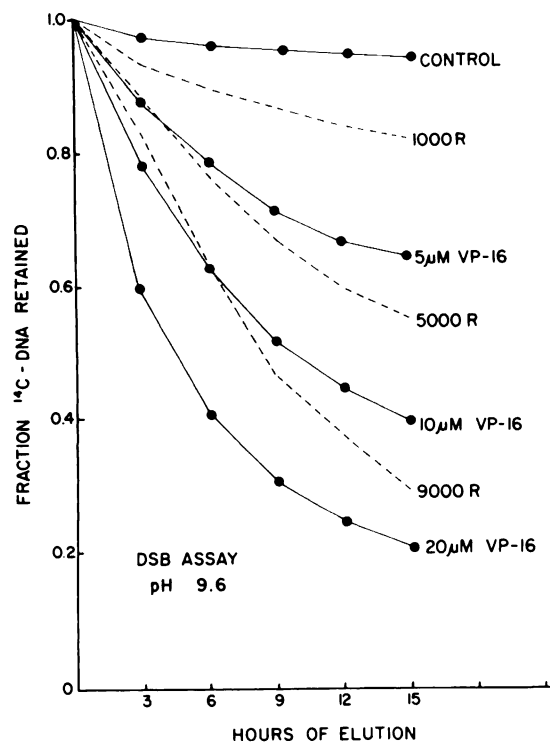


Chart 3. DNA DSBs in L1210 cells treated with VP-16 or γ -radiation. An internal standard was not used. The DNA was eluted at pH 9.6.

0°. The effect of radiation on the elution of DNA from cells not treated with VP-16 is shown as a calibration reference.

Chart 3 represents the results of the DNA DSB assay for VP-16-treated cells. Again, with subsequently higher drug doses, there were larger numbers of DNA DSBs. Cells treated with γ -radiation were again included for reference purposes.

A calibration curve for relating the frequency of VP-16-induced DNA SSBs to an equivalent effect of radiation was obtained by plotting rads *versus* ^{14}C -DNA retention at 50% retention of the internal standard (not shown). A calibration curve for DNA DSBs was obtained in a similar manner except that retention of ^{14}C -DNA after 12 hr of elution was used as the reference point. Table 1 reveals the results of converting the drug-related DNA damage to rad equivalents for drug doses ranging from 1 to 10 μM . The DSB:SSB ratio for VP-16-treated cells is consistently higher than that for cells treated with radiation.

Since many drugs which cause DNA breaks also produce other types of DNA damage, it was of interest to assay VP-16-treated cells for the presence of DNA-protein and DNA-DNA interstrand cross-links. DNA-protein cross-linking was detected by comparing the elution of DNA from cells lysed in the presence and absence of proteinase K according to a method published previously (6). This is shown in Chart 4. DNA-protein cross-linking occurred at VP-16 doses of 5 and 10 μM . Cross-linking between DNA strands was assayed by the method of Ewig and Kohn (3), and none was observed (data not shown).

It was important to determine if the effects of VP-16 on DNA occurred over a similar concentration range as that which caused cytotoxicity. Chart 5 represents the results of a soft agar colony-forming assay using L1210 cells treated with various doses of VP-16 for 1 hr. Cell survival decreased in a nearly log-linear fashion over the dose range of 5 to 60 μM . This corresponds well to the drug dose range producing DNA damage.

Since Loike and Horwitz (9) observed that VP-16 does not produce DNA damage in purified DNA, it was of interest to study the effects of VP-16 on the DNA of isolated nuclei. Charts 6 and 7 represent the data from these assays in isolated nuclei. DNA SSBs and DSBs were produced by VP-16 in nuclei over a dose range of 5 to 20 μM . Quantitative comparison of the strand break frequencies obtained in VP-16-treated whole cells *versus* isolated nuclei is confounded by the fact that internal standards were used in the assays of the former but not the latter. Nevertheless, since the total time of elution was the same in both cases, it is possible to make a rough comparison. As such, it would appear that there was only a slightly greater frequency of strand breaks occurring at a given concentration of VP-16 in whole cells than in isolated nuclei. Again, the drug-

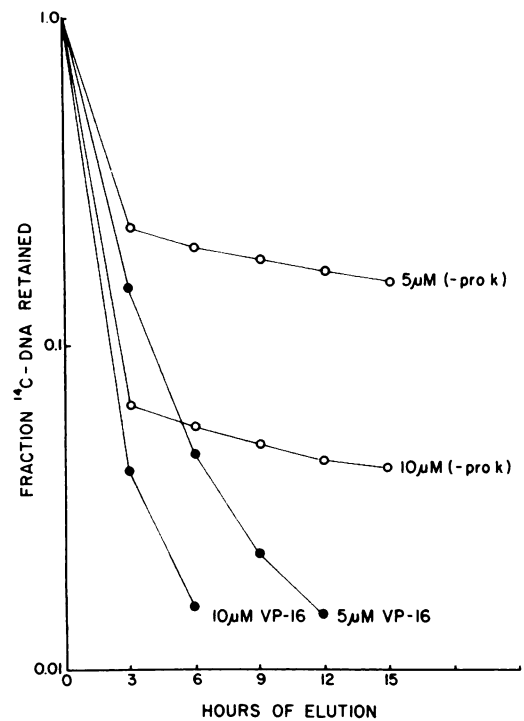


Chart 4. DNA-protein cross-linking in VP-16-treated L1210 cells. Cells lysed without proteinase K demonstrated the cross-linking while those lysed with proteinase K did not. Total elution time was 15 hr.

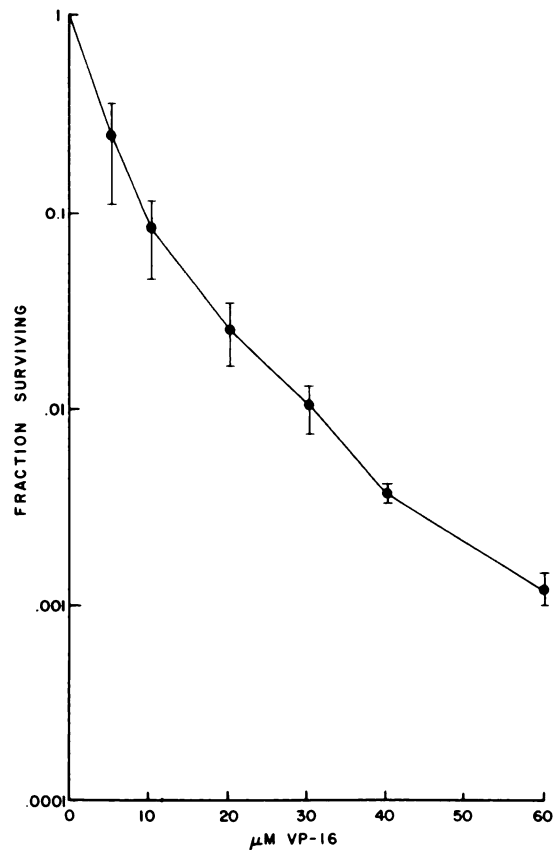


Chart 5. Colony-forming ability of L1210 cells treated for 1 hr with various doses of VP-16. The cloning efficiency of untreated cells was about 80%. Bars, S.D.

Table 1
Relationship between DNA SSB and DSB

VP-16 (μM)	SSB (rad equivalent) ^a	DSB (rad equivalent) ^a	DSB:SSB ratio
1 ^b	330 (1) ^c	700 (2)	2.1
3	880 \pm 240 ^d (3)	2470 \pm 180 (3)	2.8
5	1600 \pm 160 (6)	4720 \pm 360 (6)	2.9
10	2460 \pm 180 (5)	6550 \pm 390 (3)	2.7

^a Rad equivalents were determined as described in "Materials and Methods."

^b All drug treatments were for 1 hr.

^c Numbers in parentheses, number of experiments.

^d Mean \pm S.E.

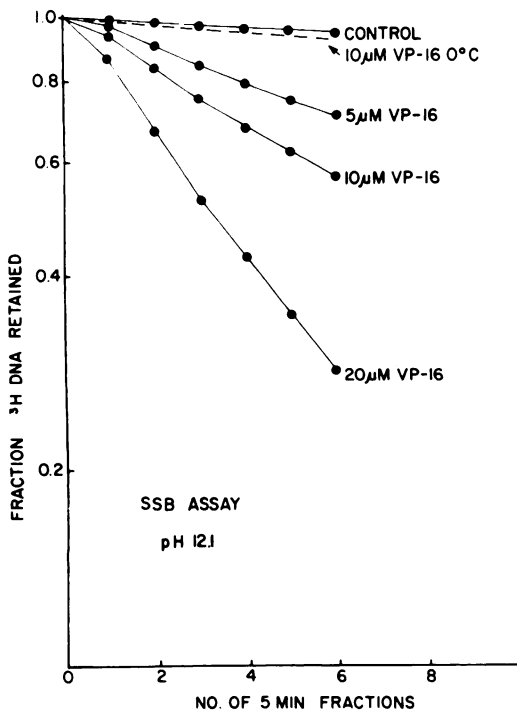


Chart 6. DNA SSBs in isolated nuclei of L1210 cells treated with VP-16. The nuclei were eluted at pH 12.1. Nuclei treated at 0° showed no SSBs. Nuclei were isolated by resuspending cells in buffer containing 0.3% Triton X-100.

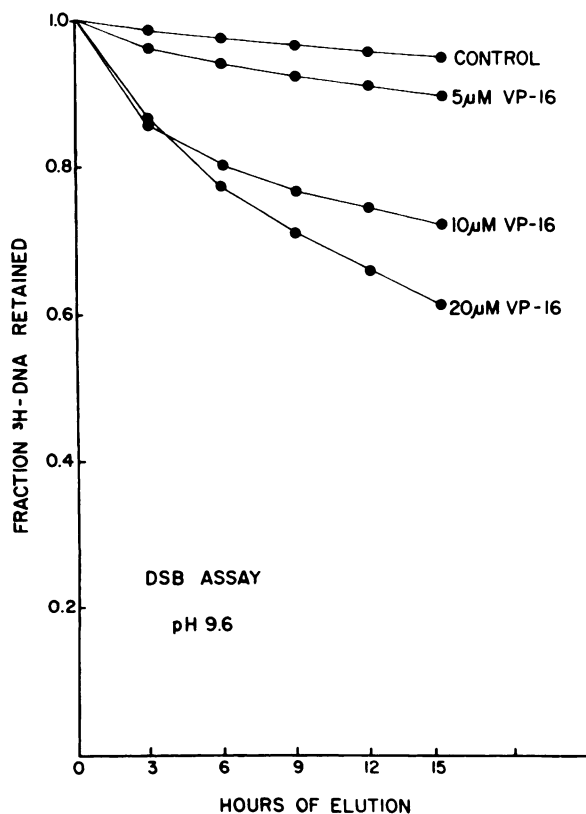


Chart 7. DNA DSBs in isolated nuclei treated with VP-16. DNA from isolated nuclei was eluted at pH 9.6.

induced DNA damage is temperature dependent, because no DNA damage occurred at 0°.

DISCUSSION

Our studies of the effects of VP-16 on the DNA of L1210 cells have confirmed the results of Loike and Horwitz (9) that cells treated with VP-16 produced DNA SSB. Our observations were made using the alkaline elution technique as opposed to the alkaline sucrose gradient sedimentation method used by the previous investigators. We have also extended their findings by demonstrating 2 additional forms of DNA damage. Both DNA DSBs and DNA-protein cross-links occur in drug-treated cells. The DNA DSBs are of special interest because they are generally considered to be more lethal lesions. The comparison of drug-induced DNA damage with radiation-induced damage allowed us to determine the DSB:SSB ratio. γ -Radiation produces a DNA DSB for every 10 to 20 SSBs that occur (7, 12). VP-16 induced more DSBs per SSB than were produced by radiation. Thus, it is unlikely that they simply represent the result of the proximity of randomly placed SSBs. We believe that these DNA DSBs may be an important factor in drug-induced cytotoxicity. As noted, DNA-protein cross-linking also occurred in drug-treated cells. These lesions can be caused by a variety of agents including alkylating agents (2), formaldehyde (11), and intercalating agents (10). While the significance of the DNA-protein cross-linking is unknown at this time, it is possible that they may eventually provide a clue to the mechanism of action of VP-16.

In addition to characterizing the types of DNA damage in drug-treated whole cells, we have explored the action of the drug on isolated nuclei. VP-16 produced both DNA SSBs and DSBs in isolated nuclei. This is of considerable interest, as Loike and Horwitz (9) were unable to demonstrate DNA damage in purified, isolated DNA exposed to VP-16. Thus, it would appear that, while cytoplasm is not necessary for drug action, there is a required component located in the nucleus, the nature of which is unknown at this time. The temperature dependence of the drug-induced DNA damage, originally observed by Loike and Horwitz (9) in cells, was confirmed by us. This temperature dependence was also seen in isolated nuclei. This indicates that it is not a function of membrane permeability but something more fundamental to drug activation, such as an intranuclear enzyme system. Isolated nuclei should provide a good model for future experimentation as they minimize the problems of membrane permeability and unknown cytoplasmic factors.

From our studies and those of Loike and Horwitz (8, 9), several important facts have emerged regarding the mechanism of action of VP-16. Cytoplasm is not required although a nuclear component of some sort is necessary. There is a temperature dependence which indicates that an enzyme system is probably involved. Finally, Loike and Horwitz (9) have shown by using various congeners of podophyllotoxin that the hydroxyl group at the C-4' position is required for activity. Based on the present extent of our knowledge, we believe that there are 2 potential mechanisms which should be investigated. First, in some fashion, the drug may activate a nuclease. This may be by binding to DNA (no current evidence exists for this) or by directly or indirectly damaging DNA, consequently activating a repair endonuclease. The second hypothesis, which

we currently favor, is that VP-16 undergoes an oxidation reduction reaction forming toxic-free radical species. This would result from the 4'OH group forming a phenoxy radical or quinone upon oxidation, thus explaining the requirement that this group be unmodified for DNA-damaging activity to occur. We speculate that an enzymatic process is involved in forming the free radical because of the temperature dependency of the reaction. The nuclei model makes it feasible to test this hypothesis by examining the action of free-radical scavengers on the effects of VP-16. Work in this laboratory is currently being directed along this path.

Finally, our paper is the first published effort to correlate VP-16-induced DNA damage with cytotoxicity. From our data, it is clear that these 2 effects occur over similar drug concentration ranges. The DNA scission is not simply the result of dying cells degrading their own DNA because the drug-treated cells do not take up trypan blue (data not shown) and because we have observed the DNA damage in drug-treated isolated nuclei. Thus, while our data do not establish that DNA damage forms the basis for drug-induced cytotoxicity, they are consistent with this hypothesis. Further studies of the relationship between these 2 phenomena are clearly indicated.

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