

## Inhibition of Etoposide-induced DNA Damage and Cytotoxicity in L1210 Cells by Dehydrogenase Inhibitors and Other Agents

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### ABSTRACT

The mechanism of action of 4'-demethylepipodophyllotoxin-9-(4,6-O-ethylidene- $\beta$ -D-glucopyranoside) (VP-16), an important antitumor agent, is unclear. There is evidence that DNA may be the target of action because VP-16 causes single-strand and double-strand breaks in DNA and produces cytotoxicity over a similar dose range. We have hypothesized that an enzyme system, such as dehydrogenase, catalyzes an oxidation-reduction reaction involving the pendant phenolic group which forms an active metabolite that causes the DNA damage and cytotoxicity. To test our hypothesis, we investigated the effect of disulfiram, an aldehyde dehydrogenase inhibitor, and its metabolite, diethyldithiocarbamate, on VP-16-induced DNA damage in L1210 cells. Using the alkaline elution technique to assay DNA damage, we found that disulfiram and diethyldithiocarbamate inhibited VP-16-induced single-strand breaks. Both compounds were also capable of significantly reducing VP-16-induced cytotoxicity. Oxalic acid, pyrophosphate, and malonic acid, competitive inhibitors of succinate dehydrogenase, and the naturally occurring dehydrogenase substrates, succinic acid,  $\beta$ -glycerophosphate, and isocitric acid, also blocked the effects of VP-16. Free-radical scavengers were also studied. While sodium benzoate was particularly effective in preventing drug-induced DNA damage and cytotoxicity, a number of other scavengers were not. Our data are consistent with the hypothesis that VP-16 is activated by an enzyme such as a dehydrogenase which transforms it into an active intermediate resulting in DNA damage and, consequently, cell death.

### INTRODUCTION

The epipodophyllotoxin VP-16<sup>3</sup> (etoposide) has become an important agent in the chemotherapeutic management of small cell carcinoma of the lung, malignant lymphomas, and germ cell tumors. Despite its established place in the clinical armamentarium, the mechanism of action of this agent is unknown. In this paper, we describe a series of experiments which provide insight into the molecular mechanism by which VP-16 acts as an anti-tumor agent.

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<sup>3</sup> The abbreviations used are: VP-16, 4'-demethylepipodophyllotoxin-9-(4,6-O-ethylidene- $\beta$ -D-glucopyranoside) (etoposide, VP-16-213); VM-26, 4'-demethylepipodophyllotoxin-9-(4,6-O-2-thenylidene- $\beta$ -D-glucopyranoside); SSB, single-strand break; DDC, diethyldithiocarbamate; DMSO, dimethyl sulfoxide; HPLC, high-pressure liquid chromatography.

Received June 6, 1983; accepted November 8, 1983.

Unlike its parent compound (podophyllotoxin), VP-16 neither inhibits microtubule assembly at the cytotoxic concentrations nor arrests cells in mitosis (10). Rather, several lines of evidence suggest that DNA may be the principal intracellular target of VP-16. Loike and Horwitz (11) and later Roberts *et al.* (13) demonstrated that exposure of mammalian cells to VP-16 or its congener VM-26 caused SSBs in DNA which were repaired after drug removal. Chromosomal aberrations have also been noted (6). We have recently reaffirmed and extended the findings of these investigators. Using the mouse leukemia L1210 cell line, we found that the dose-response relationships for cytotoxicity and DNA SSB formation were similar (14). Furthermore, additional types of DNA damage, double-strand breaks, and DNA-protein cross-links were observed. Double-strand breaks are of particular significance since they are generally considered to be more lethal than SSBs.

There are few clues to the mechanism by which DNA damage is produced by VP-16. Loike and Horwitz (11) reported that incubation of VP-16 with pure calf thymus or viral DNA did not result in strand breaks even in the presence of a reducing agent. Recent work in our laboratory, however, has demonstrated that DNA damage does occur when VP-16 is incubated with isolated nuclei and that this effect is temperature sensitive (14). It would thus appear that the necessary components for this aspect of drug activity are located in the nucleus.

In formulating a hypothesis to account for the DNA-damaging action of VP-16, we considered 2 other experimental observations. (a) The effects of DNA appear to require the phenol group on the pendant ring of the VP-16 molecule (11). Blocking this phenolic group eliminates activity. (b) VM-26, a congener of VP-16, has been shown to inhibit O<sub>2</sub> consumption in isolated mitochondria; this effect was reversed by succinate, suggesting involvement of an early step in the electron transport chain (4). Our interpretation of these findings was that the phenol group allows VP-16 to participate in some type of oxidation-reduction reaction. For example, an enzyme such as a dehydrogenase may oxidize the phenol group resulting in the formation of an active intermediate such as a phenoxy- or quinone-type radical, which would react with DNA causing strand breaks.

In order to test this hypothesis, we examined the aldehyde dehydrogenase inhibitor disulfiram and its metabolite, DDC, for their effect on VP-16-induced DNA damage in isolated nuclei and whole cells. In addition to these enzyme inhibitors, we used several other inhibitors and substrates of dehydrogenase as well as various free-radical scavengers to investigate their effects on the drug-induced DNA damage. The alkaline elution technique was used to assay DNA SSB frequency (7). Some of the various compounds were then tested for their effect on VP-16-induced cytotoxicity.

## MATERIALS AND METHODS

Mouse leukemia L1210 cells, grown in suspension in RPMI 1630 medium containing 20% fetal calf serum, penicillin, and streptomycin were used in all experiments. The doubling time was approximately 12 hr. Cells were labeled with  $[2\text{-}^{14}\text{C}]\text{thymidine}$  (53 mCi/mmol, 0.01  $\mu\text{Ci/ml}$ ; New England Nuclear, Boston, MA) or with  $[\text{methyl-}^3\text{H}]\text{thymidine}$  (20 Ci/mmol, 0.1  $\mu\text{Ci/ml}$  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, NY). It was dissolved in DMSO. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) except for SKF 525A (Smith, Kline and French Laboratories, Philadelphia, PA). All compounds except disulfiram were dissolved in media or Buffer A [1 mM  $\text{KH}_2\text{PO}_4$ ; 5 mM  $\text{MgCl}_2$ ; 150 mM  $\text{NaCl}$ ; 1 mM [ethylenebis (oxyethenenitrilo)]tetraacetic acid]. Disulfiram was dissolved in ethanol and was then added to Buffer A or media. In all instances, the appropriate solvents were added to the controls.

Isolated nuclei were prepared by washing  $^3\text{H}$ -labeled whole cells in cold Buffer A, at pH 6.4. The cells were resuspended in 1 ml Buffer A, lysed with 9 ml of Buffer B (Buffer A plus 0.3% Triton X-100; Eastman Kodak Co., Rochester, NY), and allowed to sit on ice for 30 min. Forty ml of Buffer A were added, and the nuclei were sedimented by centrifugation at 1000 rpm for 10 min. Following resuspension in Buffer A at a final density of  $10^6$  nuclei/ml, the nuclei were pretreated with the enzyme inhibitors, substrates, free-radical scavengers or cytochrome P-450 inhibitors for 15 min at  $37^\circ$ . The pretreated nuclei were then incubated with VP-16 for 1 hr at  $37^\circ$ .

DNA damage in isolated nuclei was assayed using the alkaline elution technique for high-frequency DNA SSBs (15). Nuclei containing  $^3\text{H}$ DNA were layered onto a polyvinyl chloride filter (pore size, 2  $\mu\text{m}$ ; Millipore Corp., Bedford, MA) and lysed with a solution of 2% sodium dodecyl sulfate, 10 mM disodium EDTA, and proteinase K (0.5 mg/ml; E. Merck, Darmstadt, Germany). The DNA was eluted from the filter with tetrapropylammonium hydroxide (RSA Corp., Ardsdale, NY), pH 12.1. 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.

Whole cells previously labeled with  $[2\text{-}^{14}\text{C}]\text{thymidine}$  were pretreated with disulfiram, DDC, succinate, or the free-radical scavenger for 1 hr at  $37^\circ$  at a cellular density of  $5 \times 10^6$  cells/ml. Following this, the cells were treated with VP-16 for 1 hr at  $37^\circ$ . The alkaline elution assay for DNA SSB was performed as usual except that an internal standard was used. Cells which contained  $^3\text{H}$ DNA and had received 2000 R served as the internal standard. Cells were irradiated on ice using a  $^{137}\text{Cs}$  source (Mark I irradiator; J. L. Sheppard and Associates, Glendale, CA). The rate of exposure was 2250 R/min.

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

In order to look for a nonenzymatic reaction between agents, 20  $\mu\text{M}$  VP-16 was incubated in phosphate-buffered saline with either sodium benzoate (50 mM), disulfiram (25  $\mu\text{M}$ ), succinic acid (50 mM), isocitric acid (10 mM), or with DDC (200  $\mu\text{M}$ ) for 30 min at  $37^\circ$ . The VP-16 in these fraction mixtures was then compared with VP-16 alone by a modification of a HPLC system developed by Evans *et al.* (3). A Waters Associates Radial-Pak  $\mu\text{Bondapak}$  phenyl column was used with an acetonitrile:water:acetic acid (31:68:1) solvent system at a flow rate of 6 ml/min. When sodium benzoate was used, the solvent system ratio was changed to 23:76:1. The VP-16 was detected using a Waters 440 absorbance detector at 250 nm.

## RESULTS

Disulfiram is a known inhibitor of aldehyde dehydrogenase as well as of a number of oxidases and hydrolases that are involved in oxidation-reduction reactions (2). With the premise that VP-16 is activated through an enzyme-mediated oxidation-reduction

reaction, we studied the effects of disulfiram on VP-16-induced damage in L1210 cells. Chart 1 represents the results of an alkaline elution experiment that assayed for VP-16-induced DNA SSBs when the cells were pretreated with disulfiram. At doses of 5 and 10  $\mu\text{M}$ , disulfiram affords protection in a dose-dependent fashion, with 10  $\mu\text{M}$  disulfiram giving nearly complete protection. Disulfiram itself caused a small number of DNA SSBs in the control cells. Very similar results were obtained when the effects of disulfiram on VP-16-induced DNA damage in isolated nuclei were studied (data not shown). Thus, the disulfiram effect was neither a result of preventing uptake of drug into the cell nor of a cytoplasmic interaction.

Since the relationship between DNA damage and cytotoxicity has not been established, it was of interest to learn if the disulfiram doses that protected against DNA damage in whole cells also prevented VP-16-induced cytotoxicity. The results from such experiments are shown in Chart 2. Disulfiram alone was responsible for significant cytotoxicity, as evidenced by the cloning efficiencies of the control cells. Untreated cells had a fraction survival of  $0.85 \pm 0.045$  (S.D.) as compared to  $0.45 \pm 0.082$  for 5  $\mu\text{M}$  disulfiram and  $0.032 \pm 0.015$  for 10  $\mu\text{M}$  disulfiram. In experiments in which both VP-16 and disulfiram were used, the survival was normalized to the appropriate disulfiram control. Both 5 and 10  $\mu\text{M}$  disulfiram markedly attenuated VP-16 cytotoxicity.

Disulfiram, at a concentration of 100  $\mu\text{M}$ , has been reported to act as a free-radical scavenger, inhibiting DNA cleavage caused by a variety of agents which form hydroxy radicals (9). Our studies indicate that even though 10  $\mu\text{M}$  disulfiram was capable of affording significant protection from VP-16-induced DNA damage, this same dose did not affect the amount of DNA damage

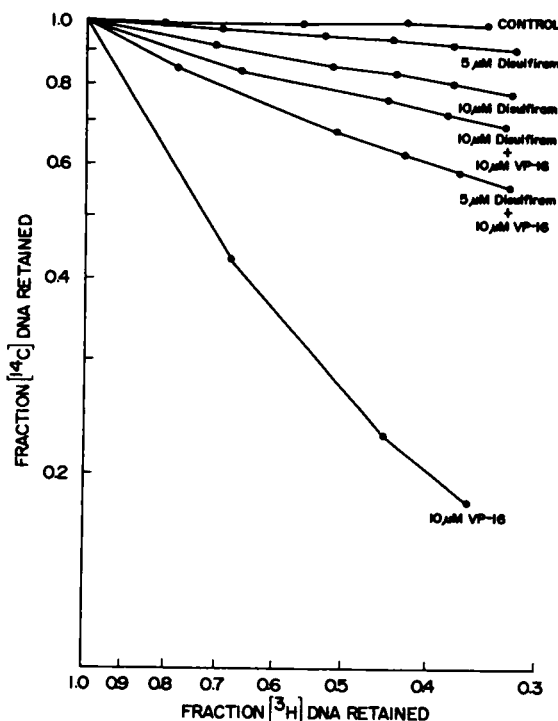


Chart 1. Effect of disulfiram on L1210 cells treated with VP-16. The cells were pretreated with disulfiram followed by a 1-hr VP-16 treatment. After resuspension in drug-free medium, the cells were lysed, and the DNA SSBs were then assayed by alkaline elution. Abscissa, retention on the filter of the internal standard  $[^3\text{H}]\text{DNA}$ . Ordinate, retention of  $[^{14}\text{C}]\text{DNA}$  from the experimental cells. The internal standard cells were irradiated with 2000 R. The DNA was eluted at pH 12.1.

caused by  $\gamma$ -irradiation (2000 R) (data not shown) which causes at least some of its DNA damage by virtue of a free-radical mechanism.

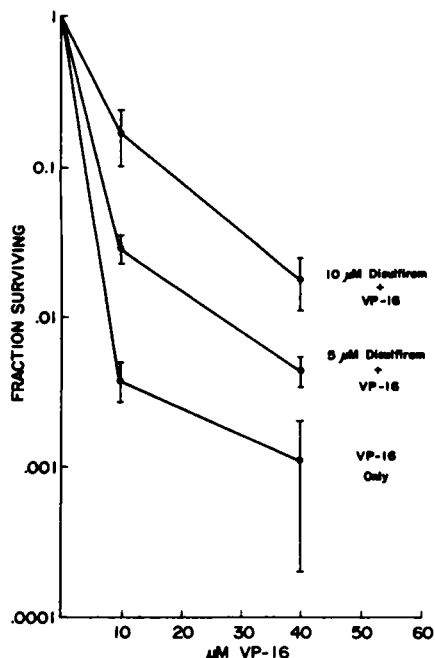


Chart 2. Influence of disulfiram on VP-16-induced cytotoxicity. L1210 cells were treated in a fashion similar to that in Chart 1. The cells were resuspended in fresh warm medium and grown in soft agar for 2 weeks. See text for cloning efficiencies. Bars, S.D.

The effects of disulfiram were shared by DDC, which is a reduced metabolite of disulfiram and presumably works by a similar mechanism (data not shown).

The effects of disulfiram supported the hypothesis that an enzyme, such as a dehydrogenase, may be responsible for activating VP-16. We thus believed that it would be useful to investigate various natural dehydrogenase substrates from the tricarboxylic acid cycle and the electron transport chain, as well as certain inhibitors of succinic dehydrogenase to see if they would be able to reverse the effects of VP-16. We hypothesized that these compounds, by competing for active sites on the enzyme that activates VP-16, would prevent drug-induced DNA damage. As evidenced by the DNA SSB assays in Charts 3 and 4, the dehydrogenase substrates isocitric acid, succinic acid, and  $\beta$ -glycerophosphate were all effective in preventing VP-16-induced DNA damage in the isolated nuclei system, as were the inhibitors oxalic acid, malonic acid, and pyrophosphate. The effects were dose dependent. Other substrates such as glutamic acid,  $\alpha$ -ketoglutarate, malic acid, and pyruvic acid were all ineffective. Of these compounds, succinic acid was tested and found to be effective as well in preventing VP-16 cytotoxicity (data not shown).

In order to test whether the effects observed with the dehydrogenase substrates were, indeed, competitive with respect to VP-16, we used the high-sensitivity alkaline elution technique which allows quantitation of DNA damage at much lower drug concentrations (7). Under these conditions, treatment of nuclei with 1  $\mu$ M VP-16 caused a significant increase in the DNA elution rate, and this effect could be completely blocked by  $\beta$ -glycerophosphate at a concentration as low as 100  $\mu$ M.

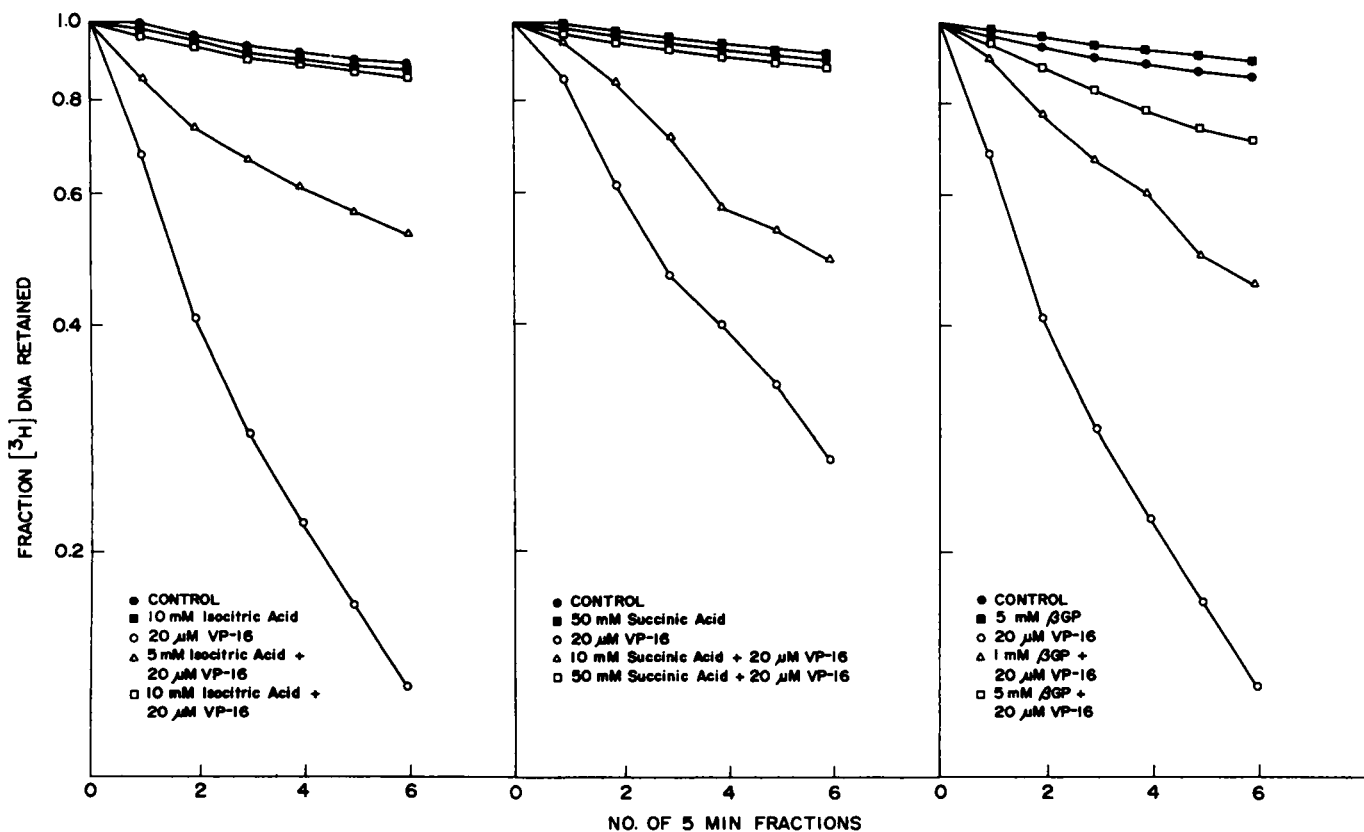


Chart 3. Effects of the natural dehydrogenase substrates, isocitric acid, succinic acid, and  $\beta$ -glycerophosphate ( $\beta$ GP) on VP-16-induced DNA SSBs in isolated nuclei. The isolated nuclei of  $^3$ H-labeled L1210 cells were treated with one of the preceding compounds for 15 min prior to a 1-hr VP-16 treatment. The nuclei were lysed, and the DNA SSBs were assayed by alkaline elution at pH 12.1.

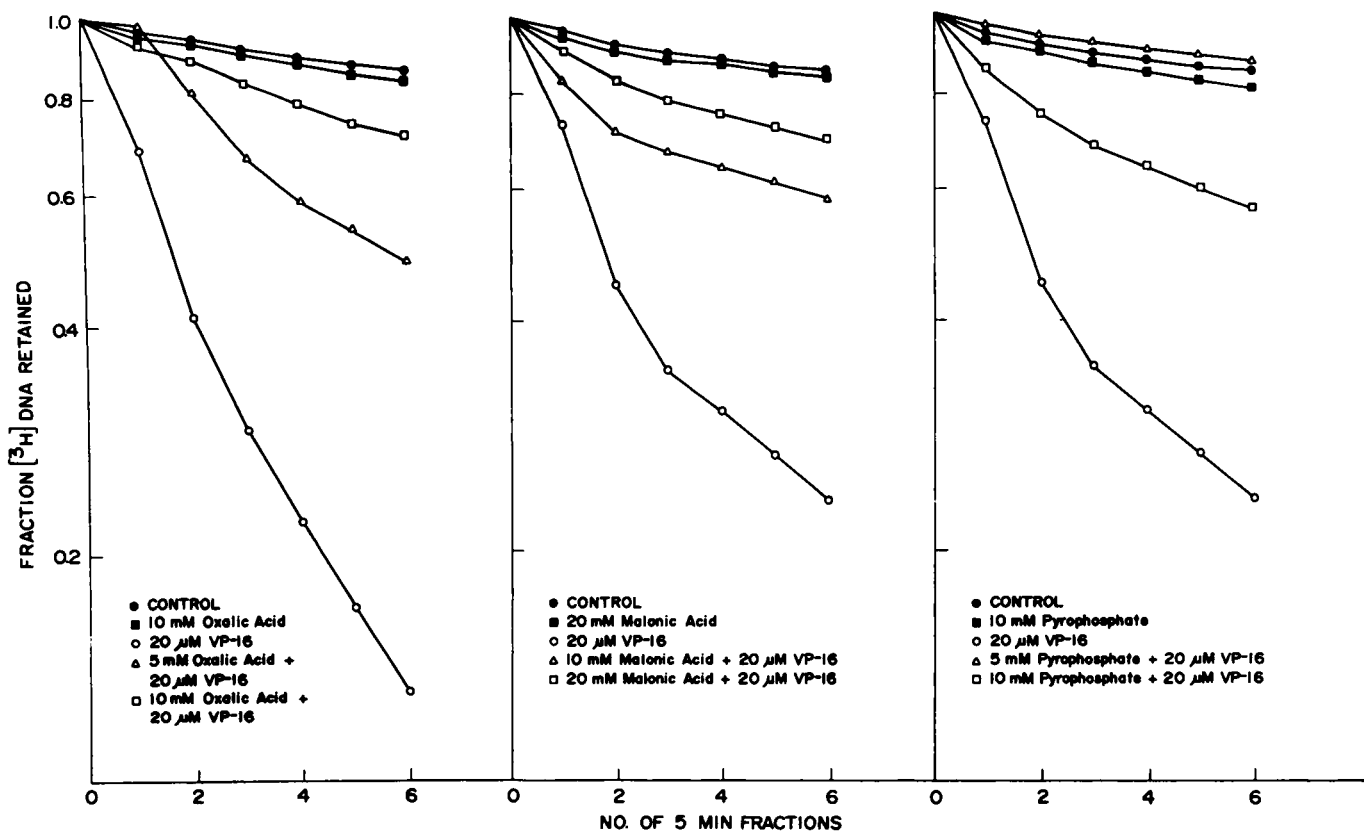


Chart 4. Effects of the succinate dehydrogenase inhibitors, oxalic acid, malonic acid, and pyrophosphate, on VP-16-induced DNA SSBs in isolated nuclei.

Both of the oxidized and reduced forms of NAD and NADP were tested for their ability to influence the activity of VP-16 in the isolated nuclei system. DNA strand breakage by VP-16 was decreased by NADPH (Chart 5) in a dose-dependent fashion, but an effect by NADP could not be consistently demonstrated. Neither NAD nor NADH had any effect on VP-16 activity.

Since we hypothesized that the actual DNA damage caused by VP-16 occurs as a result of an oxidation-reduction reaction, we studied various free-radical scavengers and their effects on VP-16-induced DNA damage. Sodium benzoate (50 to 100 mM), DMSO (1.4 M), and thiourea (50 to 100 mM) were all found to inhibit VP-16-induced DNA SSBs in isolated nuclei. In each case, the effect was dose dependent with respect to the free-radical scavenger. Many other free-radical scavengers were studied, including: superoxide dismutase (200 to 400 μg/ml); catalase (125 to 250 μg/ml); ethanol (200 mM); methanol (300 mM); mannitol (50 to 100 mM); N-acetylcysteine (1 to 100 mM); cysteamine (5 to 50 mM); dithiothreitol (2 mM); and glutathione (1 to 10 mM). All of these were ineffective in preventing VP-16-induced DNA damage (data not shown). One mechanism by which free radicals may be formed in the nucleus is via the cytochrome P-450-mediated mixed-function oxidase in the nuclear membrane. In order to test this possibility, the drug treatment of isolated nuclei was carried out in a Thundberg tube containing 80% carbon monoxide and 20% oxygen. Despite the well-known ability of carbon monoxide to inhibit cytochrome P-450-mediated reactions, no inhibition of VP-16-induced DNA scission was observed. Metyrapone and SKF 525A, other inhibitors of cytochrome P-450, were likewise without effect on VP-16 activity.

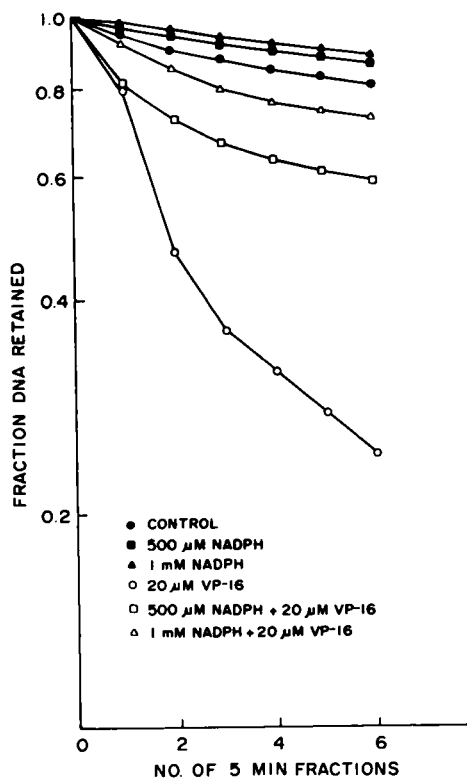


Chart 5. Effect of NADPH on VP-16-induced DNA SSBs in isolated nuclei. Nuclei were treated with NADPH for 15 min prior to a 1-hr treatment with 20 μM VP-16.

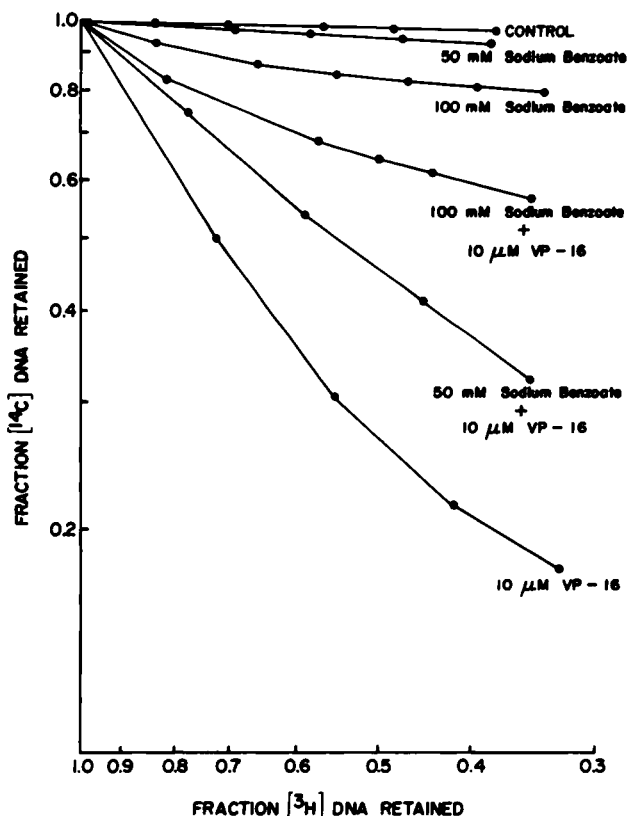


Chart 6. Effect of sodium benzoate on L1210 cells treated with VP-16. Cells containing  $[^{14}\text{C}]$ DNA were pretreated with sodium benzoate followed by a 1-hr VP-16 treatment. After resuspension in drug-free medium, the cells were lysed, and the DNA SSBs were then assayed by alkaline elution at pH 12.1.

When we examined the effect of the successful free-radical scavengers in whole cells, we found that sodium benzoate provided nearly complete protection from VP-16-induced DNA damage (Chart 6). DMSO was also used in experiments with whole cells but caused such extensive cell autolysis and DNA degradation at the concentration required in isolated nuclei that it was impossible to assess its ability to block the effects of VP-16. Thiourea (100 mM) was not protective in whole cells.

As in the case with disulfiram and succinic acid, benzoate not only protected from DNA damage but also prevented cytotoxicity. Chart 7 demonstrates the fraction of cells surviving when they were pretreated with sodium benzoate. Benzoate itself was essentially nontoxic. VP-16-induced cytotoxicity was significantly decreased in a dose-dependent fashion with respect to sodium benzoate.

In considering the mechanisms by which disulfiram, DDC, isocitric acid, succinic acid, and sodium benzoate protect cells from the effects of VP-16, we were concerned that there may be a direct interaction between these compounds and VP-16 which would render the drug inactive. Using the HPLC assay described previously, we found that neither disulfiram, DDC, isocitric acid, succinic acid, nor sodium benzoate altered the peak height or retention time of VP-16, indicating that no direct interaction takes place. In addition, we have performed similar experiments following incubation of disulfiram and VP-16 with isolated nuclei. The VP-16 peak height was again unchanged, and no new peaks were observed, indicating that disulfiram was not interacting with a VP-16 metabolite.

The alkaline elution assay is a sensitive, reproducible method for detecting and quantitating DNA strand breaks. However, the presence of strand breaks can be obscured if DNA cross-links are also present. For this reason, we have tested disulfiram, DDC, benzoate, and isocitric acid for their ability to cause DNA cross-linking. The method of Kohn and Ewig (8) was used. No cross-links were observed following exposure of isolated nuclei or of whole cells to these agents.

### DISCUSSION

On the basis of a number of experimental observations regarding the action of VP-16, we have hypothesized that VP-16 is activated via a nuclear enzyme system, such as a dehydrogenase, that allows it to undergo an oxidation-reduction reaction. This reaction produces an active metabolite which results in DNA damage and cytotoxicity. Whether the DNA strand breaks occur as a result of direct drug effect or are mediated by an endonuclease is a matter for further investigation and will not be considered further at this time. We have tested our hypothesis by using a number of compounds which might be expected to interfere with the proposed mechanism of action. In general, our observations support our hypothesis and provide some new insights into how this important chemotherapeutic agent might work.

Disulfiram and its metabolite DDC are potent inhibitors of VP-16-induced DNA damage and cytotoxicity. These compounds inhibit a number of oxidases and dehydrogenases, presumably by forming mixed disulfides at active sites on the enzymes (2). Literature values for inhibition of these enzymes are consistent with the disulfiram concentration at which the VP-16 effects are

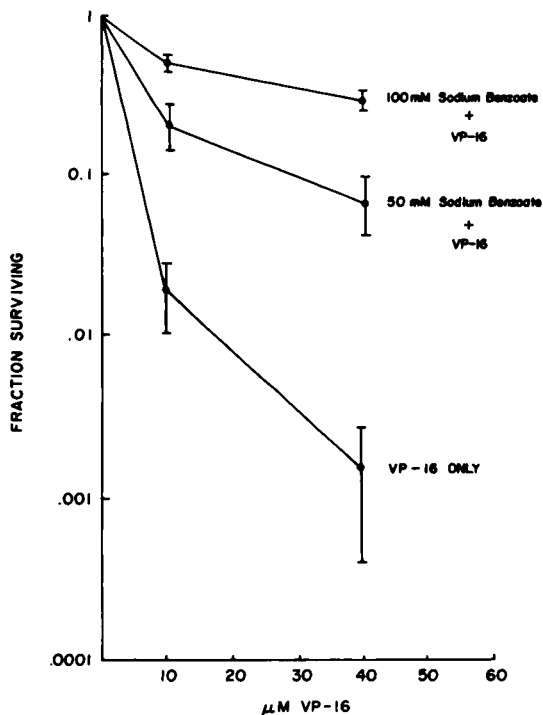


Chart 7. Influence of sodium benzoate on VP-16-induced cytotoxicity. L1210 cells were treated in a similar fashion as in Chart 6. The cells were resuspended in fresh warm medium and grown in soft agar for 2 weeks. Cloning efficiencies were: untreated cells,  $0.81 \pm .047$ ; cells treated with 50 mM sodium benzoate alone,  $0.83 \pm 0.10$ ; and cells treated with 100 mM sodium benzoate,  $0.38 \pm 0.080$ .

blocked. Not only did disulfiram inhibit the formation of DNA breaks during exposure of cells to VP-16, but also the data in Chart 2 indicate that disulfiram significantly protected cells from the cytotoxic action of the epipodophyllotoxin. We believe that the toxicity of disulfiram itself probably limited its protective effect with respect to VP-16. This toxicity is reflected in the reduced colony-forming ability and DNA strand breakage observed in cells treated with disulfiram alone. Thus, while disulfiram may prevent much of the initial damage caused by VP-16, it may, by virtue of its own toxicity, reduce the ability of the cell to recover from any residual effects of VP-16.

In addition to the effects of disulfiram, a role for a dehydrogenase-type enzyme in the action of VP-16 is supported by the results obtained using various other inhibitors and naturally occurring dehydrogenase substrates. These experiments were suggested in part by the work of Gosalvez *et al.* (4) who showed that another epipodophyllotoxin, VM-26, inhibited mitochondrial oxygen consumption, an effect which was reversed by succinate. We reasoned that, if VP-16 interrupted dehydrogenase reactions in mitochondria, perhaps under other circumstances it might serve as a dehydrogenase substrate and that, as in the Gosalvez work, some naturally occurring substrates might be able to compete for the active site. Our data support such a competitive inhibition. In considering other mechanisms by which the data shown in Charts 3 and 4 might be interpreted, it is important to note that each of the dehydrogenase substrates and inhibitors are potential chelating agents. Although the action of VP-16 requires the presence of magnesium,<sup>4</sup> it is unlikely that our observations are based on chelation for 2 reasons: (a) as in the case of  $\beta$ -glycerophosphate, the inhibitory effects can be seen at concentrations as low as 100  $\mu$ M while the magnesium concentration was 5 mM; and (b) the stability constants for magnesium and each of the substrates and inhibitors bears no relationship to their potency as inhibitors of VP-16. For instance, pyrophosphate has more than a 1000 times the affinity for magnesium as does  $\beta$ -glycerophosphate but is a weaker inhibitor of VP-16 (12). The failure of certain naturally occurring substrates to affect VP-16 activity suggests a degree of enzyme-substrate specificity, but strict interpretation will have to await further characterization of the system. In any case, we believe that our results are consistent with the concept that the action of VP-16 involves an oxidation-reduction process and is mediated by a dehydrogenase-type enzyme. Since electron micrographs of the nuclei isolated by this procedure do not demonstrate any attached mitochondria or other organelles, we believe that whatever the enzyme is it is located in the nucleus. Preliminary experiments in this laboratory indicate that the enzyme may be removed from the nuclei by extraction with 0.35 M NaCl, and current studies are directed toward isolation and purification of the enzyme.

If VP-16 must be acted on by a nuclear enzyme in order to be effective, what is the nature of the active intermediate? An inspection of the VP-16 structure would suggest that the most likely product of an interaction between the drug and a dehydrogenase is a phenoxy radical derived from the phenol group of the pendant ring. Such a radical could then directly damage DNA or generate other types of radicals which would have the same consequences. It is unlikely that oxygen radicals are involved since catalase, superoxide dismutase, and a variety of hydroxyl

radical scavengers did not affect the DNA-damaging action of VP-16. Thiourea was effective in isolated nuclei but not in whole cells. Since thiourea freely diffuses across cell membranes, it is unlikely that there was an insufficient concentration present in the nuclei of the whole cells. We think that it is more likely that the effects of thiourea on the isolated nuclei were due to an action other than radical scavenging. Sodium benzoate was highly effective both in isolated nuclei and in whole cells in blocking the effects of VP-16 (Charts 6 and 7). While this compound is widely used as a scavenger for free radicals, especially the hydroxyl radical, it has other actions at the concentrations required for the scavenging effect. Of particular interest is the observation made by Hellerman *et al.* (5) in 1946 that benzoate is a potent inhibitor of the flavoprotein component of D-amino acid oxidase. Since a variety of other free-radical scavengers were without effect on VP-16 activity, we suspect that the effects of benzoate are more probably related to direct enzyme inhibition or some other action rather than radical scavenging. Thus, at present our data do not support the production of a free-radical active metabolite of VP-16. We are currently attempting to identify products of the nuclear metabolism of VP-16 by HPLC in order to shed further light on this issue.

In summary, we have proposed a hypothesis regarding the mechanism by which VP-16 may kill cells. The effects of disulfiram, DDC, and a variety of dehydrogenase substrates and inhibitors support a role for a dehydrogenase-type enzyme, although proof of this will require isolation and characterization of this enzyme and the active drug metabolite. The correlation between inhibition of VP-16-induced DNA strand breakage and cytotoxicity leads us to believe that elucidation of the mechanism of DNA strand breakage will also provide an explanation for the antitumor effect of VP-16.

## ACKNOWLEDGMENTS

The authors wish to thank Marshall Spearman for advice and assistance in certain technical aspects of the work and Pat Strickland and Debbie Baylor for typing the manuscript. In addition, the authors wish to thank Dr. Allen Neims for many valuable discussions.

## REFERENCES

1. Chu, M. Y., and Fischer, G. A. The incorporation of <sup>3</sup>H-cytosine arabinoside and its effect on murine leukemic cells (L51784). *Biochem. Pharmacol.*, **17**: 753-767, 1968.
2. Eneanya, D. I., Blanchine, J. R., Duran, D. O., and Andresen, B. D. The actions and metabolic fate of disulfiram. *Annu. Rev. Pharmacol. Toxicol.*, **21**: 275-296, 1981.
3. Evans, W. E., Sinkule, J. E., Crom, W. R., Dow, L., Look, A. T., and Rivera, G. Pharmacokinetics of teniposide (VP-26) and etoposide (VP-16-213) in children with cancer. *Cancer Chemother. Pharmacol.*, **7**: 147-150, 1982.
4. Gosalvez, M., Perez-Garcia, J., and Lopez, M. Inhibition of NADH-linked respiration with the anti-cancer agent 4'-demethyl-epipodophyllotoxin thenylidene glucoside (VM-26). *Eur. J. Cancer*, **8**: 471-473, 1972.
5. Hellerman, L., Lindsay, A., and Bovarnick, M. R. Flavoenzyme catalysis, inhibition of D-amino acid oxidase by competition with flavin-adenine-dinucleotide of atabrine (quinacrine), quinine, and certain other compounds. *J. Biol. Chem.*, **163**: 553-570, 1946.
6. Huang, C. C., Hou, Y., and Wang, J. J. Effects of a new antitumor agent, epipodophyllotoxin, on growth and chromosomes in human hematopoietic cell lines. *Cancer Res.*, **33**: 3123-3129, 1973.
7. Kohn, K. W., Erickson, L. C., Ewig, R. A. G., and Friedman, C. A. Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry*, **14**: 4629-4637, 1976.
8. Kohn, K. W., and Ewig, A. G. DNA-protein crosslinking by *trans*-platinum (II) diamminedichloride in mammalian cells, a new method of analysis. *Biochim. Biophys. Acta*, **562**: 32-40, 1979.
9. Kuhnlein, V. Disulfiram inhibits DNA breakage by hydroxylradical-producing

<sup>4</sup> Unpublished observation.

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- agents. *Biochim. Biophys. Acta*, 609: 75-83, 1980.
10. Loike, J. D., and Horwitz, S. B. Effects of podophyllotoxin and VP-16-213 on microtubule assembly *in vitro* and nucleoside transport in HeLa cells. *Biochemistry*, 15: 5435-5442, 1976.
  11. Loike, J. D., and Horwitz, S. B. Effect of VP-16-213 on the intracellular degradation of DNA in HeLa cells. *Biochemistry*, 15: 5443-5448, 1976.
  12. Martell, A. E., and Smith, R. M. *Critical Stability Constants*. New York: Plenum Publishing Corp., 1977.
  13. Roberts, D., Hilliard, S., and Peck, C. Sedimentation of DNA from L1210 cells after treatment with 4'-demethylepipodophyllotoxin-9-(4,6-O-2-thenylidene- $\beta$ -D-glucopyranoside or 1- $\beta$ -D-arabinofuranosylcytosine or both drugs. *Cancer Res.*, 40: 4225-4231, 1980.
  14. Wozniak, A. J., and Ross, W. E. DNA damage as a basis for 4'-demethylepipodophyllotoxin-9-(4,6-O-ethylidene- $\beta$ -D-glucopyranoside)(etoposide) cytotoxicity. *Cancer Res.*, 43: 120-124, 1983.
  15. Zwelling, L. A., Michaels, S., Erickson, L. C., Ungerleider, R. S., Nichols, M., and Kohn, K. W. Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4'-(9-acridinylamino)methanesulfon-*m*-anisidide and Adriamycin. *Biochemistry*, 20: 6553-6563, 1981.