Cancer Chemother Pharmacol (1989) 23: 161–168

Cancer Chemotherapy and Pharmacology

Reduced formation of lesions in the DNA of a multidrug-resistant L1210 subline selected for teniposide resistance*

DeWayne Roberts[1], P. David Foglesong[2]*, Evangelos Parganas[1], and Lee Wiggins[1]*

[1] Division of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, Memphis, Tennessee and
[2] Biochemistry Section, Biotherapeutics, Inc., Memphis, Tennessee 38101, USA

Summary. Earlier studies have suggested that higher cellular levels of teniposide (VM-26) are required for the inhibition of growth in L1210/VM-26 sublines than in parental L1210 cells [8]. On the basis of this observation, we hypothesized that resistance to VM-26, which is partly attributed to multidrug resistance, also resulted in reduced formation of DNA lesions by the drug. In confirmation of this hypothesis, equitoxic concentrations of VM-26 produced fewer breaks in the DNA of LIa5μM cells, the prototype L1210/VM-26 subline, than in that of L1210 cells. Previously, potassium cyanide (KCN) and verapamil were shown to increase the levels of VM-26 in LIa5μM but not L1210 cells. These agents also selectively increased the formation of breaks in the DNA of LIa5μM but not L1210 cells. The DNA unknotting assay with phage P4 DNA indicated equivalent DNA type II topoisomerase activity in nuclear extracts of LIa5μM and L1210 cells. The factor that reduced the formation of breaks in cellular LIa5μM DNA by VM-26 provided less protection against equitoxic levels of doxorubicin, to which LIa5μM cells are cross-resistant.

Introduction

L1210 sublines selected in vitro for resistance to teniposide (VM-26) express many of the characteristics associated with multidrug resistance [17]. The prototype subline, LIa5μM, is 1100 times more resistant to VM-26 than parental L1210 cells and is cross-resistant to etoposide (VP-16), vincristine (VCR), doxorubicin (DOX), actinomycin D (DACT), and amsacrine (mAMSA). LIa5μM cells contain two chromosomes with homogeneously staining regions that are absent in both parental and revertant cells [7]. Although the glycoprotein associated with multidrug resistance was not detected by conventional methods in either L1210 or LIa5μM cells, a doublet of 150–155 kDA proteins that cross-react with a polyclonal antibody for the glycoprotein was detected by immunoblotting in membrane preparations of LIa5μM cells [18]. In contrast, a small, anionic protein was readily apparent in lysates of LIa5μM cells, and the addition of calcium ions to lysates resulted in a cosedimentation of the protein with subcellular fractions [17]. In the absence of calcium ions the protein remained in the supernatant fluid. This protein cross-reacted with antibody to sorcin, whose gene is in the same amplicon as the *mdr* gene in multidrug resistant sublines [18].

Changes in the plasma membrane of LIa5μM cells have also been suggested by alterations in the cellular flux of VM-26 [8], which influxed more slowly and effluxed more rapidly from LIa5μM than from L1210 cells. Steady-state levels of VM-26 were attained within 5 min and were one-fifth the level in L1210 cells; this slight difference was observed with an extracellular concentration of drug that was 900 times the IC_{50} value for L1210 cells and only 0.85 times the IC_{50} value for LIa5μM cells. The altered flux of VM-26 across the plasma membrane, cross-resistance to chemically and pharmacologically diverse drugs, the appearance of proteins that cross-react with antibody to P-glycoprotein, and the presence of sorcin in LIa5μM cells are compatible with descriptions of multidrug resistance [4, 14, 22]; however, the magnitude of these changes in LIa5μM cells appears inadequate to account solely for resistance to VM-26.

The cytotoxicity of VM-26 is attributed to an induction of single- and double-strand breaks in cellular DNA as well as DNA bound covalently to protein [19]. Similar products are formed in vitro in a DNA type II topoisomerase (TOPO II)-mediated reaction in which VM-26 stabilizes a transitional intermediate of the enzyme with DNA. Formation of TOPO II-mediated lesions in cellular DNA presumably leads to the drug-induced block of cells in the G_2 phase of the division cycle [6, 13]. This arrest in the division cycle is attenuated in LIa5μM cells, relative to L1210 cells, when the cell lines are treated with equitoxic levels of VM-26 [17].

The present results indicate that equitoxic concentrations of VM-26 produced fewer lesions in the DNA of LIa5μM cells than in L1210 DNA. A preliminary report has presented certain of these observations [16].

Methods

Cell lines. The L1210 cell lines were maintained in Eagle's minimum essential medium from GIBCO Laboratories

Offprint requests to: DeWayne Roberts, Department of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, P. O. Box 318, Memphis, TN 38101, USA

* This work was supported by Research Project Grant CA 39426 and Cancer Center Support (Core) Grant CA 21765 from the National Cancer Institute and by the American Lebanese Syrian Associated Charities

(Grand Island, NY). The powdered medium was dissolved in distilled, deionized water, autoclaved, and adjusted to pH 7.4 with 26 m*M* sodium bicarbonate plus 10% horse serum. The medium was supplemented with 2.2 m*M* L-glutamine. The LIa5μM subline was routinely maintained in medium with 5 μ*M* VM-26 until about 48 h before the study, at which time cells were transferred to drug-free medium. VM-26 was generously donated by Bristol Laboratories (Syracuse, NY) and was dissolved in dimethylsulfoxide (DMSO) before its addition to cultures. The final concentration of DMSO in the maintenance medium for LIa5μM cells was 0.03%.

Alkaline sucrose gradients. Cells were labeled by the addition of [^{3}H-methyl]thymidine, 0.8 μCi/ml (sp. act. 6 Ci/mmol), to cultures in the logarithmic phase of growth, and 18 h later either the drug or solvent for the drug was added to the cultures. The cultures were incubated further before the cells were collected by centrifugation at 1200 rpm for 5 min at 4° C. The cells were resuspended in 0.14 *M* NaCl buffered with 0.01 *M* sodium phosphate at pH 7.4 (PBS) and again collected by centrifugation. After resuspension in PBS, 1 ml cell suspension with 500,000 cells was added to 2 ml lysing solution layered on top of a gradient of 5%–20% alkaline sucrose [3]. The 30-ml gradient was formed above 2 ml alkaline 60% sucrose. After lysis for 2 h in a dark environment at 4° C, the cellular DNA was sedimented through the gradient by centrifugation at 100,000 *g* for 3 h at 4° C. Fractions of 1.1 ml were collected from the top of the gradients, after which 50 μl DNA (10 mg/ml) and 100 μl 60% trichloroacetic acid in 1.2 *N* HCl were added and the DNA was collected by centrifugation at 1200 rpm for 5 min at 4° C. The supernatant fluid was replaced with 100 μl 5% trichloroacetic acid before the DNA was hydrolyzed by heating for 10 min in a boiling water bath. The hydrolysate was transferred quantitatively to 5 ml ACS aqueous counting scintillant (Amersham Corp., Arlington Heights, Ill) for radioactivity measurements.

Alkaline elution method. For the measurement of drug-induced, single-strand DNA breaks by the alkaline elution method [5], cell lines were transferred to drug-free medium for 48 h, and at about 18 h before collection of the cells, either 0.4 μCi [^{3}H-methyl] thymidine (sp. act. 6 Ci/mmol) or 0.68 μCi 2-[^{14}]thymidine (sp. act. 34 mCi/mmol) was added per milliliter of culture. The cells were collected by centrifugation for 6 min at 400 *g* and ambient temperature and were resuspended in medium for further incubation with the drug. Portions of the cultures were transferred to polyvinyl chloride filters no. SA2J666A4 (Millipore Corp., Bedford, Mass). Cells were lysed on the filter and digested with proteinase K before the DNA was eluted with tetrapropyl ammonium hydroxide. Internal controls with irradiated cells were included with each sample, and radioactivity in the eluates was assayed with a dual label program for tritium and [^{14}C]-carbon.

Preparation of extracts with TOPO II activity. Cells from cultures in the logarithmic phase of growth were collected by centrifugation and washed twice with PBS and once with Buffer A, which consisted of 1 m*M* potassium phosphate, 5 m*M* $MgCl_2$, 150 m*M* NaCl, and 1 m*M* ethylene glycol-*bis*(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetate (EGTA) adjusted to pH 6.4 with KOH [14]. The cellular pellet was suspended in 9 volumes Buffer B, which was Buffer A with 0.3% Triton X-100 and 1 m*M* phenylmethanesulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, Mo). After the suspension was placed on ice for 30 min, 40 times the cell-pellet volume of Buffer A with 1 m*M* PMSF was added, and the nuclei were collected by centrifugation at 300 *g* and 4° C for 10 min. The nuclei were resuspended and extracted for 15 min at 4° C with 3 volumes Buffer A plus 0.35 *M* NaCl, 0.2 m*M* dithiothreitol (DTT), and 1 m*M* PMSF before collection of the nuclear extract by centrifugation for 5 min at 12,000 *g* and 4° C. The supernatant fluid was centrifuged for 1 h at 250,000 *g* and 4° C to remove ribosomal RNA. After removal of a portion of the supernatant fluid for assay of the protein content [11], the remainder was diluted with an equivalent volume of glycerol and stored at −20° C.

Preparation of phage P4 DNA. Bacteriophage P4 *vir*1 *del*[10] was generously provided by Dr. L. F. Liu. LB medium was inoculated with *E. coli* C-117, infected with phage P4, and the cultures were incubated with vigorous shaking at 37° C until the culture lysed [9]. At the completion of lysis, 10 ml 0.5 *M* EGTA and 2 ml chloroform were added per liter of culture and shaking was continued for an additional 10 min. The cellular residue was collected by centrifugation at 10,000 *g* and 4° C for 30 min. The supernatant fluid was adjusted to 0.5 *M* NaCl, 25 m*M* $MgCl_2$, and 10% polyethylene glycol (PEG 8000) and allowed to stand overnight at 4° C. The precipitate was collected by centrifugation as above and extracted three times with P4 diluent (1% ammonium acetate, 80 m*M* $MgCl_2$, and 10 m*M* TRIS-HCl, pH 7.2). The pooled supernatant fluids were centrifuged for 4 h at 100,000 *g* and 4° C, and the pellet was resuspended in P4 diluent by gentle homogenization. CsCl (0.632 gm/ml) was added and the solution was centrifuged for 18 h at 250,000 *g* and 4° C. Two opalescent bands were observed: the top band contained phage P4 and the lower band contained knotted P4 DNA. The knotted DNA was diluted to 5 ml with P4 diluent before collection by centrifugation for 3.5 h at 300,000 *g* and 4° C. The precipitate was diluted in TE buffer, 10 m*M* TRIS-HCl (pH 8.0) with 1 m*M* ethylenediaminetetraacetate (EDTA), and extracted twice with an equal volume of phenol:chloroform (1:1). The aqueous phase with the knotted DNA was then dialyzed for 24 h against four 1-l aliquots of sterile TE buffer. The quality of the knotted DNA was determined by electrophoresis through agarose gels. After electrophoresis and staining with ethidium bromide (1 μg/ml), a diffuse fluorescence of knotted DNA and a band of linear DNA were observed on the gel.

Assay of TOPO II activity. TOPO II activity was assayed by the method of Liu et al. [9]. Portions of the nuclear extracts were incubated at 37° C with 40 m*M* TRIS-HCl (pH 7.4), 100 m*M* KCl, 5 m*M* DTT, 0.5 m*M* EDTA, 0.6 μg acetylated bovine serum albumin, 1 m*M* ATP, 10 m*M* $MgCl_2$, and 1 μg knotted P4 DNA in a total volume of 20 μl. The reaction was terminated by the addition of 0.5% sodium dodecyl sulfate, after which 20 μg proteinase K was added, and the solution was incubated for 45 min at 37° C. After the addition of tracking dye, the solution was transferred quantitatively to a 0.7% agarose gel for electrophoresis. The identity of the various forms of DNA was confirmed by electron microscopy.

Results

Sedimentation of cellular DNA through alkaline sucrose gradients

Teniposide-induced lesions in DNA were measured by monitoring the rate of DNA sedimentation through alkaline-sucrose gradients. L1210 and LIa5μM cells were incubated with the drug for 1 h immediately before the cells were harvested (Fig. 1A, B). The concentrations of drug selected for these studies were equivalent to 1, 4, and 10 times the respective value for 50% inhibition of cell growth during the first 24 h after the addition of VM-26 to cultures of the two cell lines. This IC_{50} value was selected because the insolubility of VM-26 prevented the titration of cytotoxicity with LIa5μM cells when exposure to the drug was limited to 1 h. The IC_{50} value for L1210 cells was 22 n*M* VM-26 and that for LIa5μM cells was 24 μ*M* VM-26 when cultures were grown for 24 h in the presence of the drug.

Under the conditions of the assay, cellular DNA from control cultures sedimented as an aggregate that was primarily recovered in a single fraction. Breaks in the DNA of L1210 cells were present at 1 h after the addition of 22 n*M* VM-26 to L1210 cultures, and the number of breaks increased as the drug concentration was increased to 10 times the IC_{50} value. In contrast, the incubation of LIa5μM cells for 1 h with 10 times the IC_{50} value of VM-26 (240 μ*M* VM-26) produced only a small peak of more slowly sedimenting DNA, and, as expected, 24 μ*M* and 96 μ*M* VM-26 produced fewer lesions in the DNA of LIa5μM cells.

The period of incubation with VM-26 was extended to 24 h, and drug-induced DNA lesions were monitored after the addition of 88 n*M* VM-26 to L1210 cells and 96 μ*M* VM-26 to LIa5μM cells (Fig. 2A, B). In 16 studies, the growth of L1210 cultures was inhibited 75% ± 11% (mean ± SD) and that of LIa5μM cultures were inhibited 85% ± 14%. After the addition of VM-26 to L1210 cultures, the major portion of the cellular DNA sedimented more slowly than DNA from cultures treated with DMSO, the solvent for VM-26. There was less evidence of drug-induced breaks in the DNA of LIa5μM cells, with a major portion of the DNA sedimenting as an aggregate similar to DNA from cells treated with DMSO. These observations indicate that equivalent growth-inhibiting concentrations of VM-26 produced fewer breaks in the DNA of LIa5μM cells than in L1210 DNA.

Induction of lesions in LIa5μM DNA with VM-26 plus either potassium cyanide or verapamil

Potassium cyanide (KCN) increased the steady-state level of VM-26 in LIa5μM cells sevenfold but did not affect the level of drug in L1210 cells [17]. To test the effect of KCN on the production of lesions in DNA, L1210 and LIa5μM cultures were incubated for 24 h with VM-26 and then for 1 h additionally with the drug plus 1 m*M* KCN (Fig. 3). The addition of KCN to L1210 cultures treated with 88 n*M* VM-26 did not increase the number of DNA breaks produced by VM-26 alone (Fig. 3A). In contrast, the addition of 1 m*M* KCN to LIa5μM cultures with 96 μ*M* VM-26 led to the appearance of breaks that were not observed in the DNA of LIa5μM cells treated with VM-26 alone (Fig. 3B). In the absence of VM-26, the addition of KCN to LIa5μM cultures for 1 h did not alter the sedimentation rate of cellular DNA. As might be expected, the addition of 1 m*M* KCN to cultures of either cell line for 24 h caused cell lysis, irrespective of the addition of VM-26. Furthermore, breaks in the DNA of LIa5μM cells were not observed after the addition of 0.1 m*M* KCN and VM-26.

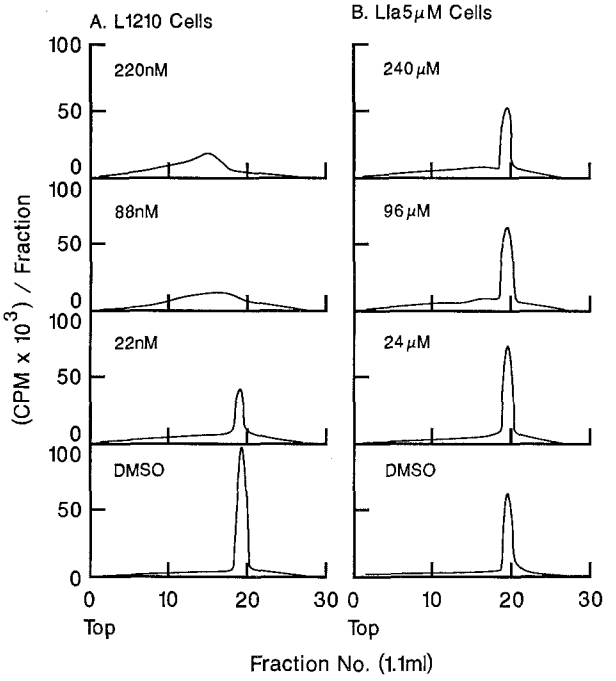

Fig. 1. Sedimentation of cellular DNA through alkaline sucrose gradients. Cultures of L1210 and LIa5μM cells were incubated for 1 h with either VM-26 or DMSO. The concentration of DMSO in the control culture was 0.4% for L1210 cells and 1% for LIa5μM cells. These concentrations of vehicle were equivalent to that added with the highest concentration of VM-26 for the respective cell line. Three hundred thousand cells prelabeled with tritiated thymidine were lysed on the gradient. After centrifugation for 3 h, the radioactivity in each 1.1 ml fraction of the gradient was assayed. Fractions were numbered from the top of the gradient

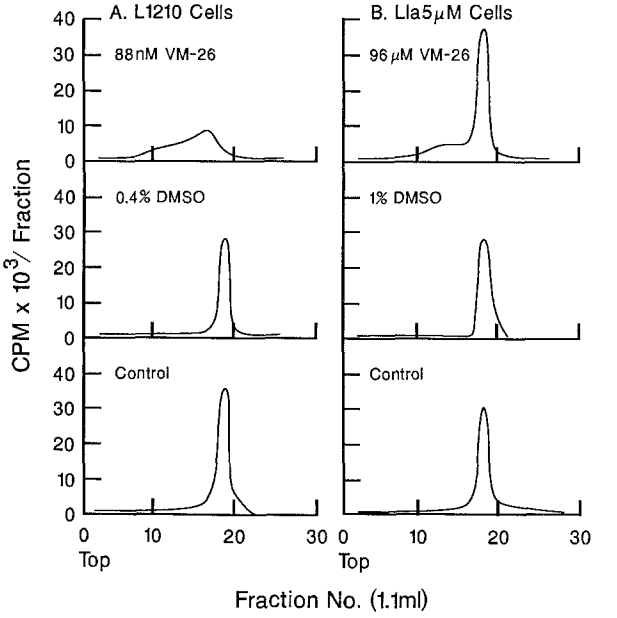

Fig. 2. The effect of VM-26 on the sedimentation of cellular DNA after incubation of L1210 and LIa5μM cells with the drug for 24 h

The addition of 10 μ*M* verapamil to L1210 and LIa5μM cultures for 1 h did not prevent the sedimentation of cellular DNA as an aggregate (Fig. 4A, B). Furthermore, the number of single-strand breaks in cellular DNA was not increased by the addition of verapamil for 1 h to L1210 cultures that were treated previously with 88 n*M* VM-26 for 24 h. The addition of 10 μ*M* verapamil to LIa5μM cultures previously incubated for 24 h with 96 μ*M* VM-26 produced breaks that were not observed in cellular DNA from cultures treated only with VM-26. In preliminary studies, the addition of 10 μ*M* verapamil to cultures for 24 h inhibited growth significantly. Concentrations of KCN and verapamil that increased the cellular level of VM-26 also increased the number of DNA lesions produced by the drug.

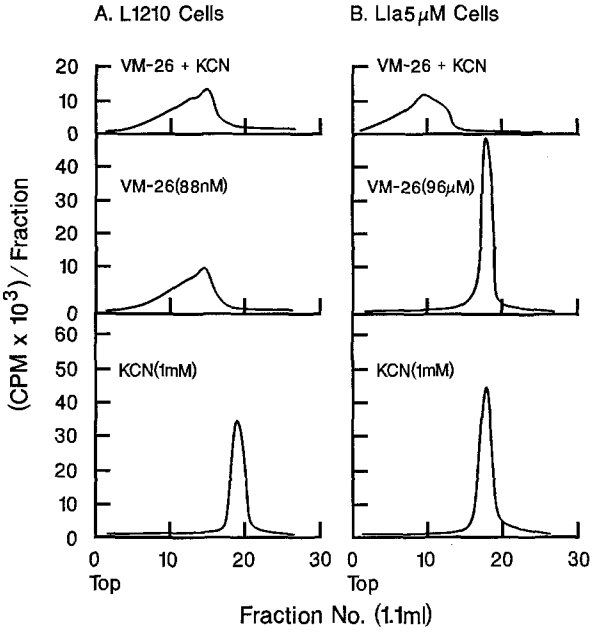

Fig. 3. The effect of KCN, VM-26, and VM-26 plus KCN on the sedimentation of DNA from L1210 and LIa5μM cells. Cultures were incubated with or without VM-26 for 24 h before KCN was added for 1 h

Induction of lesions with DOX treatment

The LIa5μM subline is 180-fold more resistant to DOX than parental L1210 cells [17], and although DOX intercalates with DNA, the drug also stabilizes the transitional intermediate of TOPO II with DNA [20]. The IC_{50} value of DOX for L1210 cells is 76 n*M*, and that for LIa5μM cells is 13.9 μ*M* [17]. Incubation of these cell lines for 24 h with a concentration of drug equivalent to 4 times the respective IC_{50} value inhibited growth by 80% and produced lesions in the DNA of both cell lines (Fig. 5). The sedimentation rate of cellular DNA was reduced slightly more for L1210 than for LIa5μM cells, and a portion of the DNA from LIa5μM cells continued to sediment at the same rate as DNA from cells in the control cultures. At equivalent growth-inhibiting concentrations, DOX produced more breaks in the DNA of LIa5μM cells than did VM-26 and about the same number of lesions in the DNA of both cell lines.

Assay of VM-26-induced lesions by the alkaline elution method

The induction of lesions in DNA by VM-26 was also assayed with the more sensitive alkaline elution method (Fig. 6). In this method, breaks in DNA result in a more rapid elution of DNA from polyvinyl chloride filters. For such studies, cultures of L1210 and LIa5μM cells were preincubated overnight with either tritiated thymidine or [^{14}C]-labeled thymidine. Residual radioactive thymidine was removed, and either VM-26 or an equivalent amount of vehicle was added to cultures prelabeled with [^{14}C]-thymidine. After additional incubation for 24 h, the cells were washed with PBS and the tritium-labeled cells were irradiated with 1500 rads. Equivalent numbers of irradiated and drug-treated cells were placed on a filter for elution of the cellular DNA. More than 95% of the [14C]-labeled DNA from L1210 and LIa5μM cells treated with DMSO remained on the filter after the elution of 70%–80% of the irradiated, tritium-labeled DNA. A significant increase in single-strand breaks occurred in the DNA of L1210 cells incubated for 24 h with 22 n*M* VM-26; however, no increase in the rate of elution was observed with LIa5μM cells treated with 24 μ*M* VM-26. When the concentrations of drug were increased

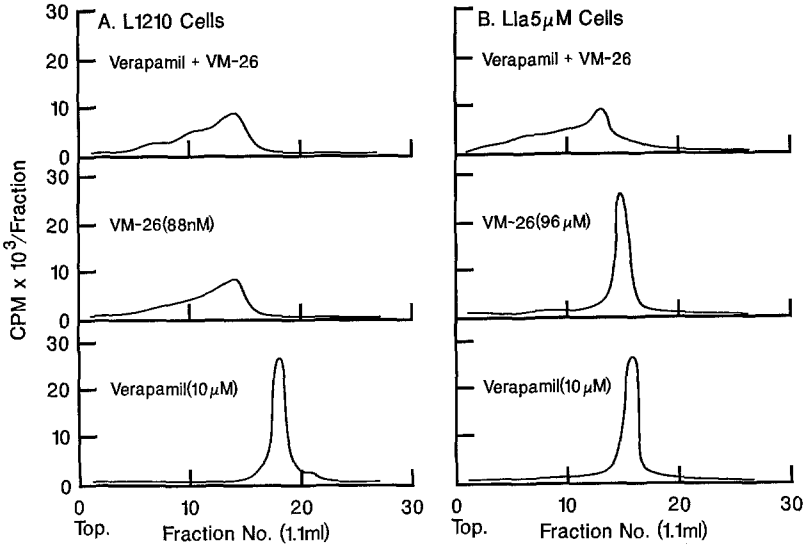

Fig. 4. The effect of verapamil, VM-26, and VM-26 plus verapamil on the sedimentation of DNA from L1210 and LIa5μM cells. Cultures were incubated with or without VM-26 for 24 h before verapamil was added for 1 h

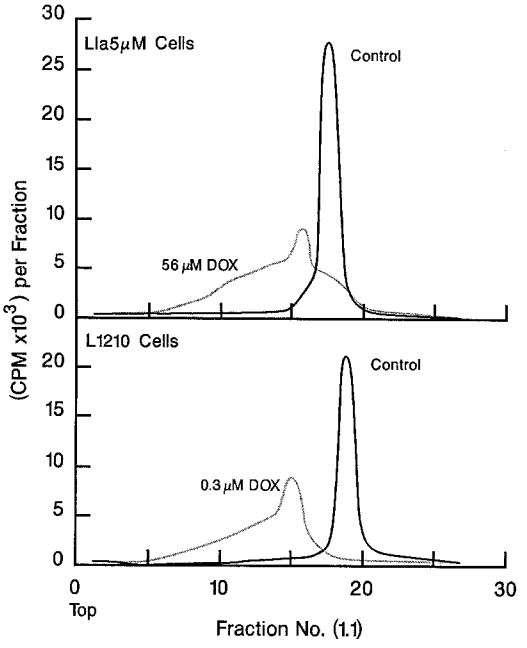

Fig. 5. The effect of DOX on the sedimentation of DNA from L1210 and LIa5μM cells. Cultures were incubated with or without DOX for 24 h before lysis of the cells and sedimentation of the cellular DNA through an alkaline sucrose gradient

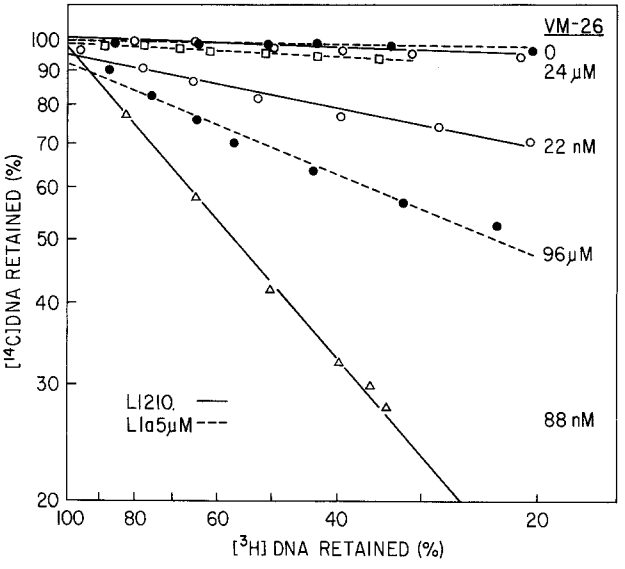

Fig. 6. The elution of cellular DNA by the alkaline elution method. Cultures of L1210 or LIa5μM cells were incubated for 24 h with or without VM-26 before the cells were collected by centrifugation at 4° C and washed twice with PBS at 4° C. Equal numbers of cells prelabeled with [^{14}C]-thymidine and of irradiated cells from control cultures prelabeled with tritiated thymidine were lysed on a polyvinyl chloride filter before the cellular DNA was eluted with tetrapropyl ammonium hydroxide. L1210 cultures were incubated with vehicle or with either 22 n*M* or 88 n*M* VM-26, whereas LIa5μM cultures were incubated with vehicle or with either 24 μ*M* or 96 μ*M* VM-26

to 4 times the respective IC_{50} values, the number of single-strand breaks in the DNA of L1210 cells increased and lesions appeared in the DNA of LIa5μM cells.

Rate constants for the elution of DNA are summarized in Table 1 for 3–6 independent studies. In the absence of

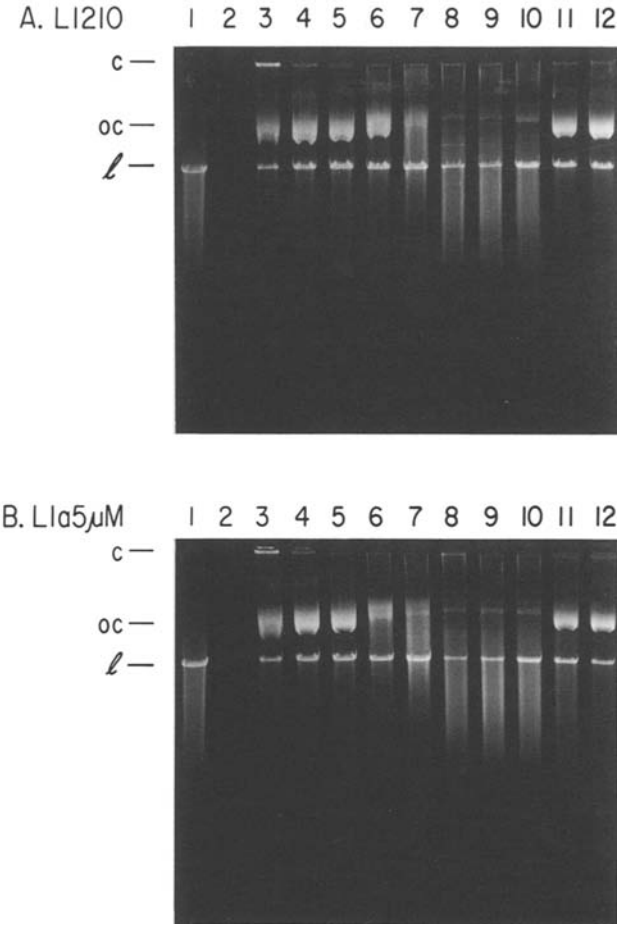

Fig. 7. TOPO II activity in nuclear extracts of L1210 and LIa5μM cells. Nuclei from L1210 (*panel A*) and LIa5μM cells (*panel B*) were extracted with 0.35 *M* NaCl solution, and the activity of TOPO II in these extracts was assayed with 1 μg phage P4 knotted DNA in the presence of 1 m*M* ATP and 10 m*M* $MgCl_2$. Products of the reaction were resolved by electrophoresis through 0.7% agarose gels. The conditions were: *Lane 1*, the nuclear extract was omitted; *lane 2*, 9 μg protein from a nuclear extract was added and the phage DNA was omitted; *lanes 3–7*, respectively, 9, 2.2, 0.56, 0.14, and 0.07 μg protein were added to the complete system; *lanes 8 and 9*, ATP was omitted and either 9 or 2.2 μg protein, respectively was added; *lanes 10–12*, 2.2 μg protein was added with 100, 10, or 1 μ*M* VM-26, respectively. The different topological forms of phage P4 DNA are catenanes (*c*), open circles (*oc*), and linear (*l*). When knotted DNA was present, it produced a diffuse fluorescence with ethidium bromide

VM-26, DNA from L1210 and LIa5μM cells eluted slowly and similarly. The addition of 22 n*M* VM-26 to L1210 cultures significantly increased the elution rate of L1210 DNA ($P < 0.01$), whereas 24 μ*M* VM-26 did not affect the elution of LIa5μM DNA. A fourfold increase in the concentration of VM-26 produced breaks in the DNA of LIa5μM cells and further increased the number of breaks in L1210 DNA. More breaks were produced in L1210 cells than in LIa5μM cells treated with much greater but equally growth-inhibiting concentrations of VM-26 ($P < 0.01$).

TOPO II activity

Equivalent levels of TOPO II activity were observed in nuclear extracts from L1210 and LIa5μM cells (Fig. 7). The P4 DNA used in this study contained both knotted and

Table 1. Slope constants for the alkaline elution of DNA

VM-26 (n*M*)	0	22	88
L1210 cells	-0.026 ± 0.003 (3)	-0.276 ± 0.157 (6)	-1.032 ± 0.187 (6)
VM-26 (μ*M*)	0	24	96
LIa5μM cells	-0.031 ± 0.016 (4)	-0.058 ± 0.02 (6)	-0.481 ± 0.13 (6)

Cultures of either L1210 or LIa5μM cells in the logarithmic phase of growth were incubated for 24 h with VM-26. Lesions in cellular DNA were determined by the alkaline elution method [17]. The concentrations of VM-26 were equivalent to either the IC_{50} value or 4 times this value, which for L1210 cells was 22 n*M* or 88 n*M*, respectively. For LIa5μM cells the concentrations were 24 μ*M* or 96 μ*M* VM-26, respectively. The integer in parentheses indicates the number of independent experiments from which the mean ± SD was derived

linear DNA, which are indicated, respectively, by the diffuse fluorescence and the band of more intense fluorescence in lane 1. The nuclear extract lacked detectable levels of nucleic acid (lane 2). With undiluted extract, the major product of phage P4 knotted DNA was catenated DNA, which remained at the origin in the agarose gel (lane 3). Dilution of the extracts led initially to the appearance of more unknotted, circular DNA and to a decrease in the amount of catenated DNA (lanes 4, 5). With further dilution of the extract, less unknotted, circular DNA was formed (lanes 6, 7) and the amount of residual knotted DNA increased concomitantly. The omission of ATP blocked the catenation and unknotting of phage P4 DNA, and residual knotted DNA was observed in both lanes 8 and 9, to which either 9 or 2.2 μg protein was added, respectively. The addition of 100 μ*M* VM-26 blocked the unknotting of P4 DNA by 2.2 μg protein from nuclear extracts (lane 10), whereas nearly normal unknotting of P4 DNA followed the addition of either 10 or 1 μ*M* VM-26 to the reaction (lanes 11 and 12). With these extracts the appearance of linear DNA was not detected, presumably because the amount of TOPO II was too low to permit the detection of this form. TOPO II activity from both L1210 and LIa5μM cells was inhibited similarly by VM-26.

Discussion

These results further indicate that resistance to VM-26 is multifaceted in L1210 cells. Earlier studies have suggested that alterations in the flux of VM-26 across the plasma membrane were unlikely to account solely for the resistance of LIa5μM cells [8]. Furthermore, those studies indicated that higher cellular levels of VM-26 are required for the inhibition of growth in L1210/VM-26 sublines than in L1210 cells. Although higher cellular levels of VM-26 are projected at equitoxic concentrations of the drug, fewer lesions were observed in the DNA of LIa5μM cells than in L1210 DNA. A similar pattern also characterized a DOX-resistant P388 subline [1, 2]. With equitoxic concentrations of DOX in the medium, cellular levels of DOX in the P388/DOX subline exceeded the level in parental cells and, as observed for VM-26 with the present L1210/VM-26 subline, fewer breaks were produced by DOX in the DNA of P388/DOX cells than in that of parental cells.

The selection of appropriate concentrations of VM-26 posed a problem. We first considered basing our studies on equivalent cellular levels of the drug in the two cell lines. This approach was discarded because of the presence of Sudan black B vesicles in 60% of the LIa5μM cells, whereas only 3% of the L1210 cells contained such vesicles [16]. In addition, resistant cells also contain other vesicles that are readily detected by phase microscopy but are absent in parental cells. Potentially, these vesicles could sequester the lipophilic drug in the resistant cells.

An alternate approach was to base the proposed pharmacological studies on a common basis of cytotoxicity for the parental and resistant lines. The original basis for cytotoxicity was the growth-inhibiting effect of VM-26 when the drug was added to cultures for 24 h. We considered reducing the time of exposure to 1 h rather than the 24-h period originally used, but the insolubility of VM-26 blocked this approach with the LIa5μM subline. We could not add sufficient drug to the medium with a 1-h exposure to inhibit the growth of LIa5μM cultures by 50% during the subsequent 24 h. Rather than switch to an alternate, less well-characterized L1210/VM-26 subline or to an intermediate period of exposure, which would have provided no substantial advantage, we elected to base the present studies on multiples of the IC_{50} values for response to a 24-h exposure. As a result, the studies in Fig. 1 were obtained after the exposure of cells for 1 h to concentrations of drug that did not produce a significant inhibition of growth during the subsequent 24 h.

A few breaks were observed in L1210 DNA within 1 h after the addition of 22 n*M* VM-26 to cultures, and the number of lesions increased as the drug concentration was increased (Fig. 1). At a concentration of VM-26 equivalent to 4 times the IC_{50} value, drug-induced breaks were observed in most, if not all, strands of cellular DNA, whereas the induction of similar lesions in the DNA of LIa5μM cells could not be duplicated by the addition of 10 times the IC_{50} value, 240 μ*M* VM-26, to cultures for 1 h.

The addition of VM-26 to L1210 cultures for 1 h produced lesions in the DNA of L1210 cells at concentrations that did not significantly inhibit cell division during the subsequent 24 h. In contrast, equivalent multiples of the IC_{50} value for LIa5μM cells did not produce a comparable number of lesions in the DNA of resistant cells. Similar results were previously observed, when lesions were produced in the DNA of L1210 ascites cells in vivo by the i.p. administration of small, nontherapeutic doses of VM-26 [15].

Although VM-26 influxes slightly slower with LIa5μM than with L1210 cells, the failure of the drug to enter the resistant sublines is unlikely to account for the absence of drug-induced lesions in the DNA of LIa5μM cells. Cellular VM-26 reaches steady-state levels within 5 min after the addition of the drug to either L1210 or LIa5μM cultures, and 95% of the drug effluxes almost as rapidly from these cells [8]. Although the lesions induced by VM-26 in the DNA of human adenocarcinoma cells disappeared with a $t_{1/2}$ value of 15–20 min after the removal of the drug [10], a minor fraction of the breaks in L1210 cells might persist longer and accumulate throughout the period of exposure

to VM-26. With the sedimentation method, no evidence for an accumulation of DNA breaks was observed when either L1210 or LIa5μM cells were incubated for 24 h with concentrations of VM-26 equivalent to 4 times their respective IC_{50} values (Fig. 2). With the alkaline elution method, a concentration of VM-26 equivalent to the IC_{50} value produced a significant number of lesions in L1210 but not in LIa5μM DNA, and at 4 times the IC_{50} value, significantly more lesions were produced in L1210 than in LIa5μM DNA. Qualitatively, these observations agree with the effect of VM-26 on the progression of L1210 cells through the division cycle [17]. About 75% of the cells were in the G_2 + M phases in L1210 cultures that received 88 n*M* VM-26, whereas 15% of the cells in control cultures and 47% of the cells treated with 22 n*M* VM-26 were in these phases at 24 h. In contrast, 24 h after the addition of 24 and 96 μ*M* VM-26 to LIa5μM cultures, only 25% of the cells were in the G_2 + M phases, whereas 14% of the LIa5μM cells in logarithmically growing cultures are normally in these two phases.

KCN and verapamil did not increase the steady-state level of VM-26 in L1210 cells but caused two- and sevenfold increases, respectively, in LIa5μM cells [16]. The increase in cellular levels of the drug is attributed to a selective inhibition of drug efflux from the resistant cells [21. In accordance with these observations, the addition of either agent to L1210 cultures that had been incubated with 88 n*M* VM-26 for the preceding 24 h did not further alter the decrease in the sedimentation rate of DNA produced by VM-26 alone. In similar studies with LIa5μM cells, 96 μ*M* VM-26 did not affect the sedimentation of cellular DNA; however, after the addition of either agent to LIa5μM cultures treated with VM-26, the sedimentation rate of DNA slowed, eventually resembling that of DNA from L1210 cells after incubation with 88 n*M* VM-26. These increases in the number of DNA breaks are attributed to increases in the cellular level of VM-26 induced by the agents. However, our earlier studies indicated that equitoxic concentrations of VM-26 produced higher cellular levels of drug in LIa5μM than in L1210 cells. A requirement for still higher levels of VM-26 to produce a significant number of lesions in DNA further suggests that the induction of lesions in DNA by the drug was altered in L1210/VM-26 cells.

Multidrug resistance originally identified resistant sublines that coincidently expressed cross-resistance to other lipophilic agents for which no common pharmacological action was known. Certain of the drugs, such as VM-26, VP-16, DOX, DACT, and mAMSA, were recently reported to interfere with TOPO II activity [19]. The LIa5μM subline is cross-resistant to these agents as well as VCR and, relative to L1210 cells, LIa5μM cells are more resistant to VCR than to VM-26 [17]. Cross-resistance to VCR is attributed to amplification of the *mdr* gene in LIa5μM cells [18]; however, resistance to VM-26 also appears to result partly from other factors that reduce the formation of DNA breaks. Cross-resistance of LIa5μM cells may result from alterations in drug flux, from a reduction in the number of lesions produced in cellular DNA, or from a combination of both factors. Since equitoxic concentrations of DOX produced similar breaks in the DNA of LIa5μM and L1210 cells, alterations in flux of the drug across the plasma membrane may also be the critical determinant of cross-resistance to this drug. If this is so, then the basis for the attenuated formation of DNA breaks in LIa5μM cells distinguishes between VM-26 and DOX.

The correlation has generally been poor between the induction of lesions in cellular DNA and the effect of VM-26 on the TOPO II activity of nuclear extracts or on purified enzyme. Much higher concentrations of the drug have been required to inhibit the enzyme in vitro than are required to stabilize the enzyme-DNA complex in cells. These requirements for greater drug concentrations for the inhibition of the enzyme in cell-free preparations might result from a localized sequestration of the drug within cells. However, if the drug is more or less uniformly distributed within the cell, the level of intracellular drug in L1210 and LIa5μM cells is only 5–10 times greater than that in the medium [8]. These concentrations of cellular drug are orders of magnitude less than the concentration required to inhibit TOPO II activity in nuclear extracts (Fig. 7). The basis for this discrepancy is unknown but may indicate that a cellular metabolite of the drug is responsible for the cellular lesion, that the association of TOPO II with chromatin increases the affinity of the enzyme for the drug, or that the growth inhibition is a much more sensitive criterion of drug action. With these important limitations in mind, we can state that no significant difference was observed in sensitivity to VM-26 between nuclear extracts from L1210 and LIa5μM cells.

K562 cells selected for resistance to VP-16 are cross-resistant to VM-26 and DOX [23]. Nuclear extracts of this subline contained only about 10% of the TOPO II activity of the parental cells, and after the removal of VP-16, lesions in cellular DNA disappeared more rapidly than in parental cells. In contrast, nuclear extracts from L1210 and LIa5μM cells had similar TOPO II activity and equivalent sensitivity to VM-26.

The present observations support our hypothesis that resistance to VM-26 in LIa5μM cells results from a variety of changes. Alterations in the flux of drug across the plasma undoubtedly contribute partly to resistance; however, an attenuated formation of DNA lesions by VM-26 apparently also contributes to resistance. Furthermore, since the growth-inhibiting effect of VM-26 does not correlate with an equivalent number of DNA lesions in sensitive and resistant cells or with equivalent cellular levels of drug, we conclude that a usually secondary action of VM-26 may be responsible for the inhibition of growth in LIa5μM cultures.

References

1. Capranico G, Dasdia T, Zunino F (1986) Comparison of doxorubicin-induced DNA damage in doxorubicin-sensitive and -resistant P388 murine leukemia cells. Int J Cancer 37: 227
2. Capranico G, Riva A, Tinelli S, Dasdia T, Zunino F (1987) Markedly reduced levels of anthracycline-induced DNA strand breaks in resistant P388 leukemia cells and isolated nuclei. Cancer Res 47: 3756
3. Friedman CA, Kohn KW, Erickson LC (1975) DNA chain growth during replication of asynchronous L1210 cells. Alkaline sedimentation studies. Biochemistry 14: 4018
4. Gerlach JH, Kartner N, Bell DP, Ling V (1986) Multidrug resistance. Cancer Surv 5: 25
5. Kohn KW, Ewig RAG, Erickson LC, Zwelling LA (1981) Measurement of strand breaks and cross-links by alkaline elution. In: Friedberg EC, Hanawalt PC (eds) DNA repair: a laboratory manual of research techniques. Dekker, New York, p 379

6. Krishan A, Paika K, Frei E III (1975) Cytofluorometric studies on the action of podophyllotoxin and epipodophyllotoxins (VM-26, VP-16-213) on the cell cycle traverse of human lymphoblasts. J Cell Biol 66: 521
7. Lee T, Roberts D (1984) Selection and characterization of L1210 sublines resistant to teniposide (VM-26). Cancer Res 44: 2981
8. Lee T, Roberts D (1984) Flux of teniposide (VM-26) across the plasma membrane of teniposide-resistant sublines of L1210 cells. Cancer Res 44: 2986
9. Liu LF, Davis JD, Calendar R (1981) Novel topologically knotted DNA from bacteriophage P4 capsids: studies with DNA topoisomerases. Nucleic Acids Res 9: 3979
10. Long BH, Musial ST, Brattain MG (1984) Comparison of cytotoxicity and DNA breakage activity of congeners of podophyllotoxin including VP16-213 and VM-26: a quantitative structure-activity relationship. Biochemistry 23: 1183
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurements with the folin phenol reagent. J Biol Chem 193: 265
12. Minford J, Pommier Y, Filipski J, Zwelling LA (1986) Isolation of intercalator-dependent protein-linked DNA strand cleavage activity from cell nuclei and identification as topoisomerase II. Biochemistry 25: 9
13. Misra NC, Roberts D (1975) Inhibition by 4′-demethylepipodophyllotoxin-9-(4,6,-0-2-thenylidene-β-D-glucopyranoside of human lymphoblast cultures in G_2 phase of the cell cycle. Cancer Res 35: 99
14. Pastan I, Gottesman M (1987) Multiple-drug resistance in human cancer. N Engl J Med 316: 1388
15. Roberts D, Hilliard S, Peck C (1980) Sedimentation of DNA from L1210 cells after treatment with 4′-demethylepipodophyllotoxin-9-(4,6-0-2-thenylidene-β-D-glucopyranoside) or 1-β-D-arabinofuranosylcytosine or both drugs. Cancer Res 40: 4225
16. Roberts D, Yalowich J, Foglesong D, Ashmun R (1986) Teniposide-resistance is multifaceted in L1210 cells. Proc Am Assoc Cancer Res 27: 267
17. Roberts D, Lee T, Parganas E, Wiggins L, Yalowich J, Ashmun R (1987) Expressions of resistance and cross-resistance in teniposide-resistant L1210 cells. Cancer Chemother Pharmacol 19: 123
18. Roberts D, Meyers MB, Biedler J, Wiggins LG (1988) Association of sorcin with drug resistance in L1210 cells. Cancer Chemother Pharmacol 23: 19
19. Ross WE (1985) DNA topoisomerases as targets for cancer therapy. Biochem Pharmacol 34: 4191
20. Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226: 466
21. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y (1982) Increased accumulation of vincristine and adriamycin in drug-resistant tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res 42: 4730
22. Van der Bliek AM, Meyers MB, Biedler JL, Hes E, Borst P (1986) A 22-kd protein (sorcin/V19) encoded by an amplified gene in multidrug-resistant cells is homologous to the calcium-binding light chain of calpain. EMBO J 5: 3201
23. Yalowich JC, Roberts D, Benton S, Parganas E (1987) Resistance to etoposide (VP-16) in human leukemia K562 cells is associated with altered DNA topoisomerase II (TOPO II) activity and rapid reversal of drug-induced DNA damage. Proc Am Assoc Cancer Res 28: 277

Received April 7, 1988/Accepted June 30, 1988