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Expressions of resistance and cross-resistance in teniposide-resistant L1210 cells*

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Summary. Resistance to teniposide (VM-26) by VM-26 selected resistant L1210 cells in culture was attributed to alterations in the flux of VM-26 across the plasma membrane and to functions of homogeneously staining regions that appeared on one or more chromosomes. In the present study, electrophoresis of membrane-cytosol fractions of these resistant sublines demonstrated a protein band, M_r 22 kd, that was not evident in similar fractions of drug-sensitive L1210 cells or three revertant sublines. The distribution of this protein among various cellular fractions could be altered by manipulation of the concentration of calcium ions. A representative subline, LIa5 μ*M*, was observed to have vesicles that reacted with Sudan black B stain, an indication of altered lipid metabolism. The LIa5 μ*M* subline was cross-resistant to etoposide, vincristine, doxorubicin, amsacrine, and actinomycin D. Concentrations of VM-26 that inhibited cell division to the same extent caused an accumulation of fewer cells in the G_2 stage of cell division in LIa5 μ*M* cultures than in L1210 cultures. These observations indicate that the LIa5 μ*M* subline expressed multiple drug resistance, as well as changes in the expression of cytotoxicity to VM-26.

Introduction

Resistant sublines can demonstrate pharmacologically important determinants for the cytotoxicity of antineoplastic drugs. The epipodophyllotoxins, teniposide (VM-26) and etoposide (VP-16), are examples of oncolytic drugs with several potentially cytotoxic actions that might be sorted for relative potency by studying resistant sublines. Although these drugs occasionally cause a transient accumulation of metaphase cells in growing cultures [9, 51, 53], more generally, progression through the division cycle is blocked either at the G_2 phase or late in the S phase near the S/G_2 interphase [13, 14, 23, 28, 34, 39, 50]. This cell-cycle-specific action of VM-26 is frequently attributed to drug-induced lesions that are expressed as breaks in cellular DNA [21, 32, 33, 44, 46, 61]. The formation of drug-induced lesions in DNA is cellularly mediated [32] and is reproduced with nuclei and with topoisomerase II in reactions that are facilitated by ATP and Mg^{2+} [10, 38, 46]. Although the cytotoxicity of VM-26 is generally attributed to these lesions, the drug also inhibits oxidative phosphorylation of Ehrlich ascites tumor cells and rat liver mitochondria, apparently by blocking NADH dehydrogenase [18]. Mitochondrial alteration was the first morphological evidence in chick embryo fibroblasts of VM-26 cytotoxicity [19] and was apparently responsible for a chronic, ultimately lethal, toxicity that followed i.p. administration of the drug to mice [2, 22, 52].

Certain characteristics of multidrug resistance [MDR] accompany the acquisition of resistance to epipodophyllotoxins. Teniposide-resistant Chinese hamster ovary (CHO cells) sublines were cross-resistant to VP-16 and 4′-demethylepipodophyllotoxin-β-*D*-glucoside but not to podophyllotoxin or other analogues of podophyllotoxin that did not produce lesions in cellular DNA [20]. Furthermore, these sublines with low levels of resistance to VM-26 and VP-16 were cross-resistant to doxorubicin, daunorubicin, ellipticine, and possibly to a number of other oncolytic agents, including actinomycin D and vinblastine [17, 20]. An etoposide-resistant subline of Ehrlich ascites tumor (EAT/VP-16) was completely resistant to VM-26 and also cross-resistant to daunorubicin and vindesine [47]. Lower cellular levels of VP-16 were attained with the EAT/VP-16 subline than with parental tumor cells, and efflux of the drug from resistant cells was energy-dependent. Resistance to VM-26 by a spectrum of L1210 sublines (L1210/VM-26 cells) correlated with a decrease in the rate of VM-26 influx and also was associated with an increase in the rate of drug efflux [31]. Cells in these sublines were characterized by one to three chromosomes with homogeneously staining regions (HSR) which were not evident in the original cell line or in revertant cells [30].

Resistance to oncolytic antibiotics and plant products is frequently associated with a variety of phenotypic expressions. These expressions include altered lipid metabolism [36, 41], changes in membrane fluidity [42, 43, 48, 58], increased levels of a large-molecular-weight glycoprotein sometimes accompanied by a reciprocal decrease in a smaller glycoprotein [6–8, 15, 16, 24, 45], the appearance

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of one or more smaller proteins in the cytosol [5, 8, 11, 26, 37], and alterations in the levels of enzymes present in the plasma membrane or involved in the turnover of glycoproteins [3, 9, 25, 40]. The large glycoprotein, M_r 170 kd, that accompanied resistance in many sublines was also associated with resistance after transfection of mouse cells with DNA from either a CHO subline that expressed MDR or a Syrian hamster subline selected for resistance to actinomycin D [45, 54]. We report that certain of these characteristics of MDR (as well as other changes that might relate more specifically to teniposide resistance), occur in the L1210/VM-26 sublines.

Materials and methods

Drugs and chemicals. Crystalline VM-26 and VP-16, gifts from Bristol Laboratories, Syracuse, NY, and from Sandoz Pharmaceuticals, Basel, Switzerland, were dissolved in dimethylsulfoxide before addition to cell culture media. Actinomycin D was a product of Merck, Sharp & Dohme, West Point, Pa, USA, Vincristine sulfate was purchased from Eli Lilly Co., Indianapolis, Ind, USA, and doxorubicin hydrochloride from Adria Laboratories, Inc., Columbus, Ohio, USA. [*Methyl*-^{3}H]thymidine (sp. act. 6 Ci/mmol) was purchased from Schwarz/Mann, Cambridge, Mass, USA. Chemicals and protein standards for electrophoresis were obtained from Bio-Rad Laboratories, Richmond, Calif, USA.

Cell culture. The L1210 cell line was graciously provided by Isodore Wodinsky, Arthur D. Little Co., Inc., Cambridge, Mass, USA. L1210 cells and their variants were maintained as stationary suspension cultures in Auto-Pow (Eagle's minimum essential medium) with 10% horse serum and *L*-glutamine (Flow Laboratories, McLean, Va, USA. The L1210/VM-26 sublines were selected by increasing the concentration of VM-26 in the medium and were maintained in medium with a concentration of VM-26 equivalent to that in the soft agar used for cloning [30]. About 48 h before study, the L1210/VM-26 sublines were transferred to drug-free medium. All cell lines were maintained at 37 °C in an atmosphere with 5% CO_2. The method of Bauer [4] was used to stain cells with Sudan Black B. Steady-state cellular levels of VM-26 and rates of drug efflux were determined as reported previously [33].

Gel electrophoresis. About 20 million cells from cultures in either the middle or the late phase of exponential growth were collected at room temperature by centrifugation at 100 *g* for 8 min and washed twice with PBS (0.15 *M* NaCl solution buffered with 0.01 *M* sodium phosphate at pH 7.5). The cell pellet was dissolved at 4 °C in 150 µl 1% nonidet NP-40) in PBS solution containing 2 m*M* phenylmethylsulfonyl fluoride (PMSF). The insoluble material was removed by centrifugation at 12000 *g* for 2 min, and the supernatant fluid was diluted with an equal volume of Laemmli buffer [29]. The content of protein in the samples was measured with the Bio-Rad protein assay. These membrane-cytosol fractions were stored at −20 °C until the samples were prepared for electrophoresis by heating at 100 °C for 5 min. The cellular proteins and standard proteins were applied to 3.5% polyacrylamide stacking gels and resolved by electrophoresis through gradient slab gels of 5%–15% polyacrylamide [29]. Gels were stained with Coomassie Brillant Blue R-250, and the size of proteins was estimated by the method of Weber and Osborn [57].

Fractionation of cells. In other studies 0.2 ml pellets of LIa5µ*M* cells were resuspended in 20 volumes of a hypotonic solution composed of 10 m*M* *Tris* (hydroxymethyl)-aminomethane (TRIS), 0.8 m*M* $MgCl_2$, 0.8 m*M* PMSF, 12 m*M* iodoacetate, and 8 m*M* 2-mercaptoethanol at pH 8.0. At times this solution was supplemented with 1 m*M* $CaCl_2$. After 30 min at 4 °C, the plasma membranes were ruptureded by forcing the cell suspension through a 23-gauge needle with a 10-ml syringe. Nuclei were collected by centrifugation at 2000 *g* for 10 min, and the supernatant fluid was diluted to make a 0.25 *M* sucrose solution in 10 m*M* TRIS and 1 m*M* PMSF at pH 8.0. A second fraction was collected by centrifugation at 10000 *g* for 10 min. The supernatant fluid was then centrifuged for 1 h at 100000 *g* to collect the third fraction. Protein remaining in the supernatant fluid was precipitated with 5% trichloroacetic acid and collected by centrifugation at 17000 *g* for 20 min. Each pellet was resuspended in an equivalent volume of double-strength Laemmli buffer before electrophoresis. Trichloroacetic acid in the fourth fraction was neutralized wth NaOH prior to electrophoresis.

Cell kinetics. After incubation of cultures for 24 h with or without VM-26, 1 million cells were collected by centrifugation at 160 *g* for 5 min at room temperature. The cells were resuspended in a hypotonic solution of propidium iodide, 50 µg/ml in 0.1% sodium citrate [27]. Cells were treated with 2 µg RNase (Calbiochem, La Jolla, Calif, USA) per ml suspension for 30 min at room temperature immediately before analysis by flow cytometry. The stained cells were analyzed at a rate of approximately 300 cells/s with an EPICS 753 flow cytometer (Coulter Corp., Hialeah, Fla) after excitation with a Coherent Innova 90 argon ion laser adjusted to deliver 500 mW at 488 nm. Fluorescence from propidium iodide-stained cells was measured at wavelenghts greater than 570 nm, and the fluorescence of approximately 100000 cells was recorded. The data were transferred directly to a Data General MV/8000 minicomputer, and the percentage of cells in the G_1, S, and G_2+M phases of the cell cycle were determined by analysis with the computer program PEAK provided by Dr. Philip N. Dean [12].

Results

Characteristics of LIa5µM cells

Table 1 shows the properties of the LIa5 µ*M* subline that indicate a drug-resistant phenotype [30, 31]. LIa5 µ*M* cells were over 1000 times as resistant to growth inhibition by VM-26 than were the parental L1210 cells. Although modal chromosome numbers were identical in the resistant and parental cell lines, the former contained homogeneously staining regions, a hallmark of drug resistance due to gene amplification. One such region was on a submetacentric chromosome [30]. Alterations in the flux of drug across the plasma membrane were indicated by a decreased rate of drug influx and by an increased rate of efflux. As a result, steady-state cellular levels of VM-26 were lower than in L1210 cells, and these lower levels may have resulted in the observation of less nondiffusible drug.

Table 1. Characteristics of acquired resistance to VM-26

Property	L1210 cells	LIa5μM cells
IC_{50} value (μ*M* VM-26)[a]	0.022	24
Medium (μM V*M*-26)	0	5
Population doubling (h)	16	17
Chromosomes		
Modal no.	40	40
Mean no.	41	41
Homogeneously staining regions	0	2
Submetacentric	0	1
Influx rate (pmol VM-26/10^7 cells/min)[b]	1380	360
Efflux rate constant (min^{-1})	0.21	0.38
Steady state level (pmol VM-26/10^7 cells)	1530	340
Nondiffusible drug (pmol VM-26/10^7 cells)	106	27

[a] IC_{50} value is the level of VM-26 that must be added to the medium to inhibit growth by 50% for 24 h

[b] Assayed in phosphate-buffered saline with 20.5 μ*M* VM-26

Table 2. Cross-resistance of LIa5μM cells to oncolytic drugs

Drug	IC_{50} values[a]		R values
	L1210 (n*M*)	LIa5μM (μ*M*)	
VM-26	22.0± 1.0 (22)	28.60±0.60 (3)	1300
VP-16	180.0±30.0 (3)	38.50±1.80 (3)	214
Vincristine	1.4± 0.1 (5)	2.10±1.10 (5)	1500
Adriamycin	76.0±35.0 (3)	13.90±3.70 (3)	183
Actinomycin D	2.5± 0.8 (3)	1.49±0.70 (3)	596
Amsacrine	21.0± 3.0 (4)	0.87±0.16 (4)	41

Cultures of either L1210 cells or LIa5μM cells were grown in various concentrations of drug for 24 h. The inhibition of growth by the various drugs is expressed as both the IC_{50} value and the R value (e.g., the concentration of drug required to inhibit growth by 50% and the ratio of these values for the two cell lines, respectively)

[a] Mean ± SD; the number of observations is given in parentheses

Cross-resistance of LIa5μM cells

The LIa5 μ*M* subline was cross-resistant to VP-16, vincristine, doxorubicin, actinomycin D, and amsacrine (Table 2). An unexpected finding was that LIa5μ*M* cells were more resistant to vincristine and actinomycin D than to etoposide, which both structurally and pharmacologically is much more similar to VM-26. Furthermore, unlike the other drugs vincristine has not been shown to interfere with topoisomerase II [10, 55]. The cross-resistance of LIa5μ*M* cells to these drugs is consistent with the pattern of MDR established with other cell lines and drugs.

Protein composition of L1210/VM-26 sublines

Alterations in protein composition are frequently associated with MDR, although a consistent pattern of change has not been recognized. Membrane-cytosol fractions of the LIa5μ*M* subline, as well as from eight other L1210 sublines with various levels of resistance to VM-26, contained a small protein of $M_r \approx 22000$ (Fig. 1, lanes 2–10). A similar band, however, was not observed in preparations of L1210 cells (lane 1) or of revertant sublines (lanes 11–13) that were obtained by serial passage of resistant sublines (lanes 2–4, respectively) in drug-free medium for 1 year [30]. Resistance to VM-26 was associated with the appearance of a protein that was not apparent in membrane-cytosol preparations of either L1210 or revertant cell lines.

In an attempt to identify the cellular location of the 22-kd protein, various cellular fractions from LIa5μ*M* cells were isolated by differential sedimentation (Fig. 2). If calcium ions were added to the lysates, a 22-kd protein was found in sediments obtained after centrifugation at 2000 *g*, 10000 *g*, and 100000 *g* and could be precipitated from the remaining supernatant fluid by the addition of trichloroacetic acid. As expected, a similar protein was not observed in corresponding fractions from L1210 cells. If calcium ions were omitted, a 22-kd protein was only observed in the fraction precipitated by trichloroacetic acid. If calcium ions were added after centrifugation at 2000 *g*, a

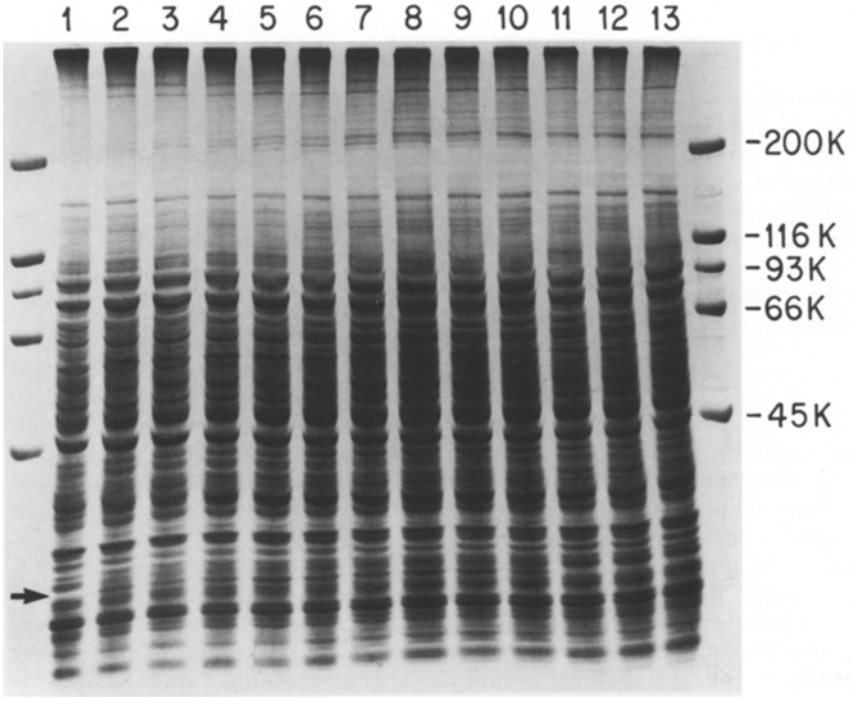

Fig. 1. Electrophoresis of membrane-cytosol fractions from sensitive, VM-26-resistant, and revertant L1210 cell lines. Membrane-cytosol fractions, 48 μg protein per lane, from the various cell lines were resolved by electrophoresis and stained with Coomassie Blue. Selection of the cell lines was described earlier [30]. The sources of proteins and relative resistance to VM-26 of the various L1210 sublines (in parentheses) were: lane *1*, L1210 (1); lane *2*, LIa5 μ*M* (1100); lane *3*, LIb5 μ*M* (2045); lane *4*, LIc5μM (1680); lane *5*, LIa2 μ*M* (770); lane *6*, LIc2 μ*M* (1000); lane *7*, VIc (770); lane *8*, VId (770); lane *9*, HI (182); lane *10*, LI7.5 (140); *11*, LIa5DF (4); lane *12*, LIb5DF (5); lane *13*, LIc5DF (10). The unnumbered lanes contain protein standards with the approximate molecular weight indicated on the right. The *arrow* indicates the position of a protein ($M_r \approx 22$ kd) that was present in the resistant sublines but was not observed in similar fractions from parental cells or revertant cells

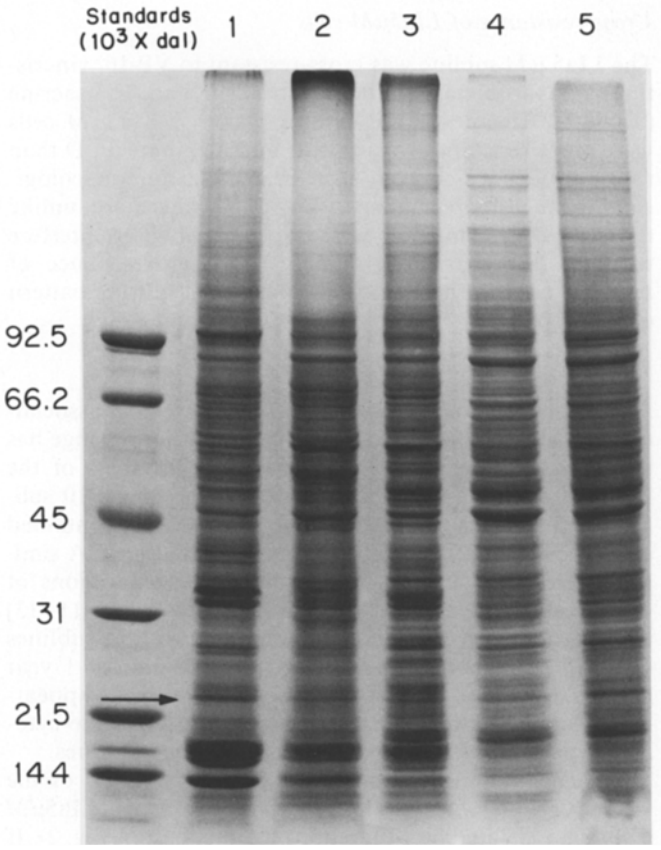

Fig. 2. Distribution of the 22-kd protein among subcellular fractions. LIa5 μ*M* cells were ruptured, and cellular fractions were collected as described in *Materials and methods.* The hypotonic solution contained 1 m*M* $CaCl_2$. The samples for lanes *1, 2,* and *3* were sedimented by sequential centrifugation at 2000 *g*, 10000 *g*, and 100000 *g*, respectively. The material in lane *4* remained in solution after centrifugation at 100 *g* and was precipitated with 5% trichloroacetic acid before resuspension n Laemmeli buffer. Lane *5* is an internal control of LIa5 μ*M* cells that were prepared as described in the legend to Fig. 1. The *arrow* indicates the position of the 22-kd protein

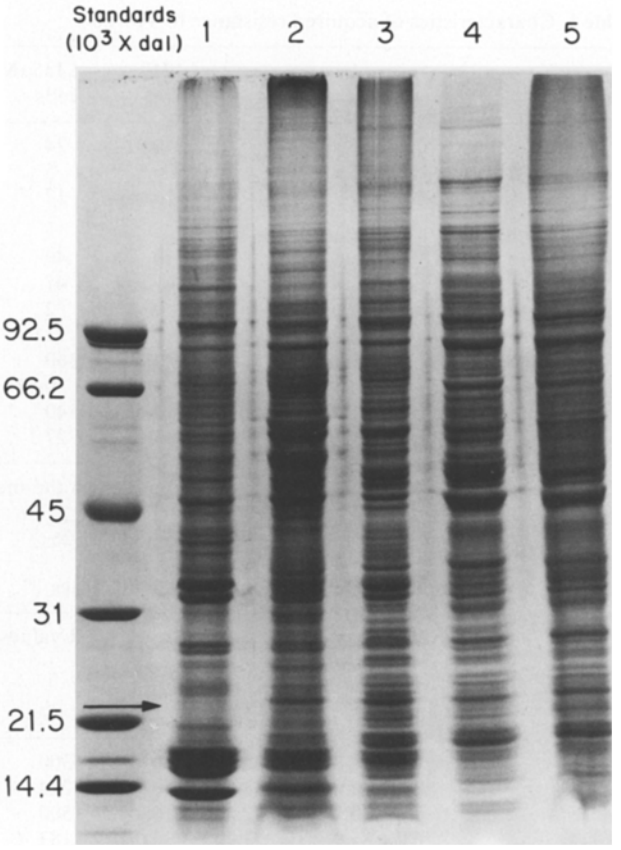

Fig. 3. Distribution of the 22-kd protein with delayed addition of $CaCl_2$. LIa5 μ*M* cells were ruptured in hypotonic solution that lacked $CaCl_2$, and the nuclei were collected by centrifugation at 2000 *g* (lane *1*). The supernatant fluid was diluted with 0.25 *M* sucrose solution containing 2 m*M* $CaCl_2$. Cellular fractions sedimenting at 10000 *g* (lane *2*) and at 100000 *g* (lane *3*) were collected sequentially. Proteins in the supernatant fluid were precipiated with 5% trichloroacetic acid (lane *4*). An aliquot of a membrane-cytosol fraction from LIa5 μ*M* cells was included as a control (lane *5*). The position of the 22-kd protein is indicated by an *arrow*

22-kd protein was present in the three other fractions, but not in the nuclear fraction (Fig. 3). Conversely, if 6 m*M* ethyleneglycol-bis(β-aminoethylether)tetraacetic acid (EGTA) was added to solutions after collection of the nuclei, a 22-kd protein was observed both in the nuclear fraction and in the trichloroacetic acid-precipitated fraction but nor in the fractions sedimented at 10000 *g* or 100000 *g* (Fig. 4). These observations indicate that calcium ions are required for binding of the 22-kd protein to certain cellular fractions.

Lipid pools in LIa5μM cells

By phase microscopy, the L1210/VM-26 sublines were observed to contain more cells with cytoplasmic vesicles than the L1210 cell line. Sixty percent of the cells in logarithmically growing LIa5μ*M* cultures contained vesicles that were stained by Sudan Black B, whereas only 3% of the cells in L1210 cultures contained such vesicles (Table 3). The proportion of cells with lipid droplets and the number of these vesicles per cell were the same irrespective of whether the LIa5μ*M* cell line was cultured in medium with 5 μ*M* VM-26 or transferred to drug-free medium for 48 h before the study. Selection of L1210 cells for resistance to VM-26 produced a subline in which there was a greater incidence of cells with lipid vesicles.

Effect of verapamil and cyanide on VM-26 levels

Cellular levels of agents can be increased in MDR sublines by combining drugs with verapamil or inhibitors of oxidative phosphorylation [49, 56, 60]. The addition of either 10 μ*M* verapamil or 1 m*M* potassium cyanide to LIa5 μ*M* cultures increased cellular steady-state levels of VM-26 twofold or sevenfold, respectively (Table 4). Neither agent, however, affected cellular levels of VM-26 in L1210 cells. The magnitude of the stimulation by verapamil and cyanide varied for different studies but was observed consistently.

In five experiments, the twofold enhancement in cellular level of VM-26 produced by verapamil was not associated with a significant decrease in the rate of VM-26 efflux ($P=0.45$, paired *t*-test). In contrast, preliminary experiments indicate that potassium cyanide caused at

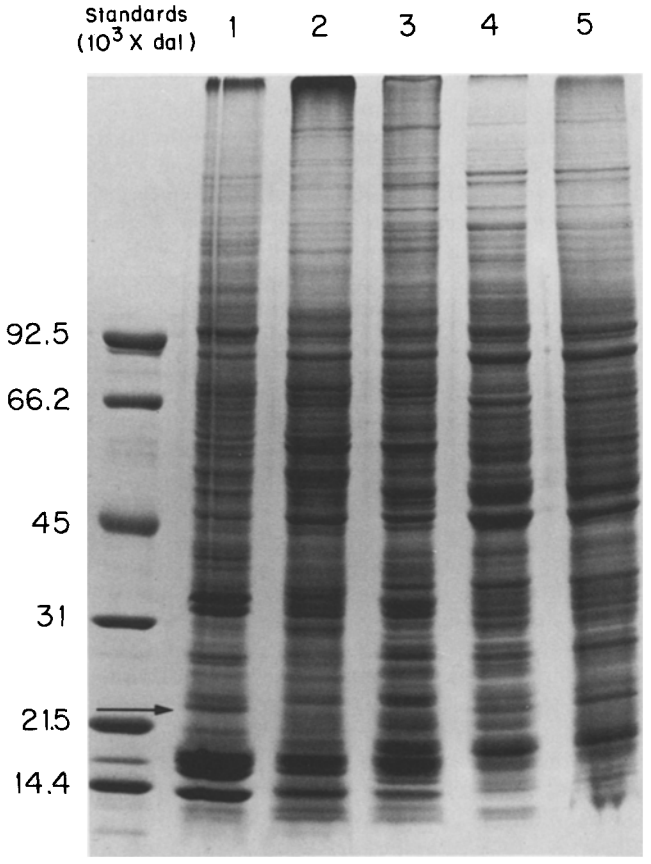

Fig. 4. Distribution of the 22-kd protein after chelation of Ca^{2+} with EGTA. LIa5 μ*M* cells were ruptured in a hypotonic solution with 1 m*M* $CaCl_2$, and the nuclei were collected by centrifugation at 2000 *g* (lane *1*). The supernatant fluid was diluted with 0.25 *M* sucrose that contained 6 m*M* EGTA before sequential centrifugation at 10000 *g* (lane *2*) and at 100000 *g* (lane *3*). Proteins in the supernatant fluid were precipitated with 5% trichloroacetic acid (lane *4*). As a control, an aliquot of a membrane-cytosol lysate of LIa5 μ*M* cells was resolved in lane *5*. The position of the 22-kd protein is indicated by an *arrow*

least a threefold decrease in the unidirectional rate constant for VM-26 efflux. The growth of L1210 and LIa5μ*M* cultures was inhibited by the addition of 10 μ*M* verapamil, and growth inhibition was additive for both L1210 and LIa5μ*M* cells when 10 μ*M* verapamil was combined with VM-26 at a concentration equivalent to the corresponding IC_{50} value. Furthermore, the addition of lower concentrations of verapamil to cultures did not increase the sensitivity of LIa5μ*M* cells to VM-26.

Cell cycle inhibition

Normally, about 45% of the cells in logarithmically growing L1210 and LIa5μ*M* cultures are in the G_1 stage of the division cycle, and the addition of 5 μ*M* VM-26 to LIa5μM cultures did not significantly alter the distribution of cells among the stages of the division cycle (Table 5). Routinely, LIa5μ*M* cells are cultured in this concentration of drug to prevent reversion. However, the addition of 22 n*M* VM-26 to L1210 cultures or of 24 μ*M* VM-26 to LIa5μ*M* cultures for 24 h inhibited cell division about 65% and increased the proportion of cells in the G_2+M stages from about 15% to about 50% for L1210 cultures and to 23% for LIa5μ*M* cultures. Concentrations of drug equivalent to 4 times the IC_{50} value, i.e., 88 n*M* VM-26 for L1210 cells and 96 μ*M* for LIa5μM cells, inhibited the growth of cultures by about 95% and increased the proportion of cells in the G_2+M stages to 75% for L1210 cultures and to 25% for LIa5μ*M* cultures. Equivalently growth-inhibitory concentrations of VM-26 were less effective in blocking the progression of LIa5μ*M* cells through the G_2+M stages than in blocking L1210 cells.

Table 3. Number of Sudan Black B-stained vesicles

	Cell lines		
	L1210	LIa5μM	LIa5μM
μ*M* VM-26	0	0	5
Cells scored	300	300	298
Number with vesicles	10	181	179
Vesicles/cell[a]	1.8 ± 1.03	3.67 ± 1.95	3.00 ± 2.09

L1210 cells and LIa5μM cells from cultures in the log phase of growth were stained with Sudan Black B, and 100 cells from each culture were scored for the presence of lipid vesicles. One culture of LIa5μM cells was transferred from medium with 5 μ*M* VM-26, in which the cell line is generally maintained, to drug-free medium for 48 h prior to staining. The observations were combined from three similar studies

[a] Mean ± SD

Table 4. The effect of verapamil or cyanide on steady-state cellular levels of VM-26

VM-26	Cell line	
	L1210 (%)	LIa5μM (%)
10 μ*M* Verapamil	108 ± 8[a] (4)	210 ± 47 (6)
1 m*M* KCN	93 ± 10 (4)	684 ± 304 (5)

Values are recorded as percentage change in steady-state, cellular levels of [^{3}H]VM-26 relative to controls for each cell line. For each experiment three to five measurements were made of steady-state levels

[a] Mean ± SD with number of experiments indicated in parenthesis

Table 5. The effect of VM-26 on the distribution of cells within the division cycle

Cell line	VM-26 (μ*M*)	Growth inhibition[a] (%)	Division cycle stage[a]		
			G_1 (%)	S (%)	G_2+M (%)
L1210					
	0	–	42 ± 3	43 ± 4	15 ± 2
	0.022	69 ± 7	17 ± 2	36 ± 5	47 ± 4
	0.088	93 ± 3	5 ± 2	19 ± 4	75 ± 5
LIa5μM					
	0	–	45 ± 5	41 ± 7	14 ± 2
	24	64 ± 2	41 ± 3	36 ± 3	24 ± 1
	96	94 ± 2	40 ± 16	34 ± 12	26 ± 6
	5	–	48 ± 5[b]	36 ± 6[b]	15 ± 1[b]

The distribution of cells among the various stages of the division cycle was determined by fluorescence-activated flow cytometry

[a] Values are means ± SD for three observations unless indicated otherwise

[b] Mean ± SD for two studies

Discussion

The observation of cross-resistance to vincristine, doxorubicin, actinomycin D, and amsacrine indicates that resistance to VM-26 was accompanied by MDR. MDR is generally attributed to alterations in the flux of drugs across the plasma membrane and quite often to expression of an energy-dependent system for the efflux of drugs from cells. This efflux is blocked by verapamil as well as by inhibitors of oxidative phosphorylation [49, 56].

The rates of VM-26 influx and efflux were altered in the spectrum of L1210/VM-26 cell lines from which the LIa5 μ*M* subline was taken, and the rates of VM-26 influx correlated inversely with resistance of the sublines to VM-26 [31]. With 20.5 μ*M* VM-26 extracellularly, the rate of VM-26 influx into LIa5μ*M* cells was one-fourth as fast as for L1210 cells, and efflux of the drug was twice the rate for parental cells. With this fixed concentration of extracellular VM-26, steady-state levels of VM-26 were lower in L1210/VM-26 cells than in L1210 cells, but varied independently with respect to the level of resistance [31]. For LIa5μM cells the steady-state levels of VM-26 were about one-fifth the level in L1210 cells. With LIa5μ*M* cells the level of VM-26 was doubled by the addition of 10 μ*M* verapamil and was increased sevenfold by 1 m*M* potassium cyanide, whereas these agents did not affect the level of VM-26 in L1210 cells. Qualitatively, the differential action of these agents on drug efflux from LIa5 μ*M* sublines is also characteristic of MDR.

Changes in the flux of VM-26 most probably resulted from alterations in the plasma membrane, since metabolites of VM-26 were not detected in cells after the brief, 20-min studies of drug transport [31]. This suggestion is further supported by the rapid loss of VM-26 from L1210/VM-26 cells at 4 °C, whereas L1210 cells effectively retained drug with similar conditions. The 20-fold increase in the incidence of LIa5 μ*M* cells with lipid vesicles may also relate to alterations in the lipid composition of the plasma membrane, since lipid vesicles and changes in the lipid composition of the plasma membrane were observed with murine P388 leukemia cells selected for resistance to adriamycin [41].

The appearance of a 22-kd protein in L1210/VM-26 cells further indicates an association between resistance to VM-26 and MDR. This protein was not detected in L1210 cells or revertant sublines and may be analogous to proteins observed in drug-resistant sublines of Chinese hamster lung fibroblasts, mouse tumor cells, and human tumor lines selected for resistance to vincristine [5, 8, 26, 35, 37]. These proteins varied in size from 19 kd to 30 kd. In certain instances the location of the protein was traced to the cytosol, while in others it was associated with the plasma membrane. The 22-kd band from LIa5μ*M* cells apparently does not contain galactose in a terminal or penultimate position, since it was not labeled by tritiated sodium borohydride in the galactose oxidase method, irrespective of pretreatment with neuraminidase. This observation may indicate a cytosolic location for the protein; however, we are uncertain of this, because the presence of the protein in various cellular fractions could be changed by altering the concentration of calcium ions in solutions used for their isolation. Meyers and Biedler suggest that such proteins are products of amplified genes in vincristine-resistant sublines [36]. The 22-kd protein observed by Koch et al. [26] had a high affinity for calcium ions, and membrane-associated protein observed by Marsh and Center was phosphorylated [35]. The association of homogeneously staining regions with appearance of the 22-kd protein in L1210/VM-26 cells supports their suggestion.

Alteration of the glycoprotein composition of the plasma membrane frequently accompanies the selection of sublines with MDR. Such changes include increased amounts of a protein with an M_r of 170 kd, which is occasionally associated with decreased amounts of glycoprotein with an M_r of 100 kdul. It has been suggested that a 170-kd protein relates to the altered flux of drug in MDR [24]. Although we cannot exclude the presence of the 170-kd protein in LIa5 μ*M* cells, it was not detected with tritiated sodium borohydride labeling of galactose in the terminal or penultimate positions of proteins or with two-dimensional electrophoresis of [^{35}S]methionine-labeled proteins (Lee and Roberts, unpublished observation).

Another change that may be more specifically related to the pharmacological action of VM-26 than to MDR was observed with LIa5μ*M* cells. The cytotoxicity of 4′-demethylepipodophyllotoxins is generally attributed to drug-induced lesions in DNA. With VP-16, a congener of VM-26, formation of single-strand breaks increased in proportion with increases in drug concentration and cytotoxicity [59]. Presumably, the drug-induced lesions in DNA that are produced by both VP-16 and VM-26 and lead to these breaks are also responsible for the G_2 stage block in the progression of cells through the division cycle [28, 39]. With LIa5 μ*M* cells, the association of cytotoxicity with a G_2 block was attenuated, and this attenuation occurred with concentrations of drug that should have produced higher cellular levels of VM-26 than in L1210 cells treated with a comparably cytotoxic concentration of VM-26.

In the studies of drug transport, the extracellular concentration of VM-26 was 20.5 μ*M* and resulted in the accumulation of about 4.5 times more drug in L1210 cells than in LIa5 μ*M* cells. This extracellular concentration of VM-26 is 800 times greater than the IC_{50} value for L1210 cells and only about 0.85 times the IC_{50} for LIa5μM cells. Since VM-26 enters L1210 cells by passive diffusion [1], we conclude that much larger cellular levels of VM-26 are required to inhibit LIa5 μ*M* cells than L1210 cells, and suggest that the cytotoxicity of VM-26 for LIa5 μ*M* cells results from what is usually a secondary action of the drug. Such a shift in the basis for the cytotoxicity of VM-26 would indicate that resistance is also associated with an alteration in the usual, primary site of drug action. In summary, certain acquired phenotypic expressions of LIa5 μ*M* cells resemble those reported for MDR, while other changes apparently are related more specifically to the cytotoxicity of VM-26.

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