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Multidrug resistance in a small cell lung cancer line: rapid selection with etoposide and differential chemosensitization with cyclosporin A

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We developed a multidrug resistant small cell lung cancer line, VPR-2, by exposing H69 parent cells to etoposide (20 μM) for 1 h daily for 3 days every 21–28 days, a schedule similar to that used in the clinic. Resistance (20-fold) to the cytostatic and DNA cleavage activities of etoposide emerged after the third treatment, and this phenotype was stable in the absence of drug exposure for 2.5 years. VPR-2 cells exhibited cross resistance to intercalating agents and vinca alkaloids, but remained sensitive to X-radiation, cisplatin and 5-fluorouracil. The human *mdr1* gene was overexpressed in the resistant line, but steady-state concentrations of etoposide were reduced only 1.5-fold. Topoisomerase II catalytic and etoposide stimulated DNA cleavage activity in nuclear extracts from both lines were identical despite retention of a 3-fold level of resistance to etoposide-induced strand breaks in isolated nuclei from VPR-2 cells. Cyclosporin A and verapamil, both of which bind to P-glycoprotein, enhanced accumulation of etoposide in VPR-2 cells, and H69 cells to a lesser extent. Yet only cyclosporin A was effective in differentially enhancing etoposide cytostasis in VPR-2 relative to H69. In VPR-2 whole cells, cyclosporin A enhanced etoposide-induced DNA single-strand break frequency 9-fold but had no effect in isolated nuclei. Rapid selection of this line with a clinically relevant drug exposure schema and stability of the resistant phenotype suggest these cells may have been a steady subpopulation of the parent line through years of serial passage *in vitro*. The data also indicate that the ability of cyclosporin A to enhance etoposide sensitivity in VPR-2 cells is unlikely to be mediated wholly through an interaction with P-glycoprotein. Since cyclosporin A, but not verapamil, served to discriminate VPR-2 cells from the parent line, this effect may offer a clue to an additional mechanism of resistance in this cell line.

Key words: Cyclosporin A, etoposide, multidrug resistance, small cell lung cancer.

Introduction

It is obvious that multidrug resistance represents a major obstacle to progress in cancer treatment. However, the mechanisms of the resistance encountered clinically remain mostly obscure. Small cell lung cancer is particularly enigmatic in this regard. At presentation, this disease typically responds dramatically to a variety of drugs, but the tumor eventually proves broadly resistant and fatal in the majority of patients. Clearly this broad chemoresistance does not correspond to any one *in vitro* model of multidrug resistance and is probably related to several factors, both host and tumor-related.

In vitro models using tumor cell lines have identified some of the mechanisms which can contribute to multidrug resistance such as overexpression of the P-glycoprotein plasma membrane drug efflux pump, qualitative and quantitative changes in DNA topoisomerase II, and increased glutathione content or increased activity of related enzymes that enhance detoxification of xenobiotics and free radicals. Within one cell line, multiple mechanisms frequently contribute to the resistant phenotype and, from clinical observations, these cell lines may be the most relevant. Specifically as regards small cell lung cancer, all of the mechanisms detailed above have been implicated in cell lines selected *in vitro* under a variety of drug exposure schemes.[1–4] However, those schemes frequently bear little or no resemblance to the way in which the drugs are used in the clinic and yet likely dictate to a large extent the mechanism that is selected for or induced. We now report initial characterization of a multidrug resistant small cell lung cancer line (VPR-2) that represents our effort to address this problem and develop a more clinically relevant model for drug resistance. We used a relevant drug for this tumor

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(etoposide) and a drug exposure scheme modeled on common clinical usage. We observed relatively rapid selection of a resistant phenotype (as opposed to longer schemes which are likely to induce as well as select). The VPR-2 cell line is of particular interest; although the cells overexpressed P-glycoprotein relative to the parent line, only cyclosporin A, and not verapamil, differentially sensitized the resistant cells to etoposide. Our findings indicate that factors in addition to P-glycoprotein expression contributed to drug resistance in VPR-2 cells and suggest that chemosensitization mediated by cyclosporin A can occur through multiple paths.

Materials and methods

Chemicals

The following chemicals were used: culture medium, fetal calf serum, glutamine and antibiotics (Gibco, Grand Island, NY); ITS (insulin, transferrin and selenous acid) supplement (Collaborative Research, Bedford, MA); [^{3}H]etoposide, 900 mCi/mmol (Moravek Biochemicals, Brea, CA); [^{3}H]daunomycin, 4.0 Ci/mmol (New England Nuclear, Boston, MA); [^{3}H]cyclosporin A, 11 Ci/mmol (Amersham, Arlington Heights, IL); vinblastine, vincristine and verapamil (Sigma Chemical Co., St Louis, MO); tetrapropyl ammonium hydroxide (RSA Corp., Ardsdale, NY); etoposide and cisplatin were a gift from Bristol Laboratories (Syracuse, NY); mitoxantrone a gift from Lederle Laboratories (Pearle River, NY); and daunomycin a gift from Dr Leonard Zwelling (UTMDACC, Houston, TX). 4′ - (9 - Acridinylamino)methanesulfon - *m* - anisidide (*m*-AMSA) was obtained from the National Cancer Institute (Bethesda, MD). Cyclosporin A was provided by Sandoz (Basle, Switzerland). 11-methyl-leucine-cyclosporin A was provided by Dr Philip Durette (Rathway, NJ) and FK506 was donated by Fujisawa Pharmaceutical Co. (Osaka, Japan). Etoposide and *m*-AMSA were dissolved in dimethyl sulfoxide. Cyclosporin A was dissolved in absolute ethanol. The remaining drugs were dissolved in water.

Cell lines and culture techniques

The H69 line was developed by Carney *et al.*[5] from a patient with small cell lung cancer and was purchased from American Type Tissue Culture Collection (Rockville, MD). The VPR-2 line was established from H69 cells as detailed in Results. Both lines grow in suspension in tight cellular aggregates with doubling times of 30–40 h. Both lines were cultured in RPMI 1640 with 2.5% fetal calf serum supplemented with ITS [insulin (5 mg/ml), transferrin (5 mg/ml) and selenous acid (5 mg/ml)] and PSN [penicillin (50 mg/ml), streptomycin (50 mg/ml) and neomycin (100 mg/ml)]. Mycoplasma testing was performed every 6 months (Bionique Labs, Saranac Lake, NY). Cells were counted by passing an aliquot repetitively through a small bore pipet tip to produce a single cell suspension. Viable cells were counted with a hemocytometer after staining with trypan blue.

Growth inhibition

A modification of the dimethyl-thiazol-diphenyl-tetrazolium bromide (MTT) assay was used to measure drug-induced cytostasis.[6] This is a colorimetric assay dependent upon the ability of viable cells to reduce MTT to a blue formazan product. Cells were treated for 1 h with drug at 37°C with phosphate buffered saline, and seeded in 96-well plates at 1×10^4 cells per well in sextuplet for each drug dose. On day 6, 25 μl of MTT (5 mg/ml) was added and plates incubated for 2 h. After addition of 100 μl extraction buffer [20% sodium dodecyl sulfate (SDS) dissolved in 50% *N*,*N*-dimethyl-formamide], plates were incubated overnight. Absorbance at 570 nm was read on day 7 with a Dynatech MR600 plate reader (Dynatech, Chantilly, VA). Cytostatic effect was calculated as percentage reduction in absorbance relative to control after correction for background. Using small cell lung cancer lines, this assay is highly reproducible and correlates well with the results of clonogenic assays in a previous comparative study.[7]

Alkaline elution

Single-strand breaks in DNA were assayed by alkaline elution as previously described.[8] Cells were labeled with [^{14}C]thymidine for 48 h and grown in label-free media for 24 h prior to drug exposure for 1 h. Internal standard cells that contained [^{3}H]thymidine-labeled DNA and that had received 150 or 1500 rad prior to elution were included on each filter. High-frequency single-strand breaks were assayed at an elution rate of 0.16–0.20 ml/min with a total elution time of 30 min. Low-frequency single- and double-strand breaks were measured by eluting at 0.03–0.04 ml/min for 15 h.

Drug transport

Steady-state concentrations of [^{3}H]etoposide, [^{3}H]daunomycin and [^{3}H]cyclosporin A were measured by incubating 2×10^7 cells with drug for 60 min at 37°C. Cells were then processed as described previously[9] and values expressed as nanomoles of drug per gram dry cell weight.

Isolated nuclei and nuclear extracts

Nuclei from both cell lines were isolated as previously described[10] in 0.3% Triton-X 100 (Eastman Kodak Company, Rochester, NY). Isolated nuclei were used immediately in drug treatment for alkaline elution studies or were further extracted in 0.35 M sodium chloride for topoisomerase II assays, as described.[11]

Preparation of kinetoplast DNA (kDNA)

kDNA was purified from Sarkosyl extracts of *Crithidia fasciculata* trypanosomes by cesium chloride–ethidium bromide density gradient centrifugation as described.[12] kDNA was labeled with [^{3}H-methyl]-thymidine by adding 1 mCi of the isotope to 300 ml of growing trypanosome cultures 24 h before purification.

Type II topoisomerase assays

Quantitative analysis of decatenating activity in 0.35 M sodium chloride nuclear abstracts was assayed using [^{3}H]kDNA, as described previously.[13,14] Drug-stimulated DNA cleavage activity was assayed qualitatively by the generation of form III (linearized) DNA from supercoiled form I SV40 DNA as detailed previously.[11]

Chemosensitization

Cells were incubated with cyclosporin A and verapamil for 1 h prior to and during the hour of drug exposure unless otherwise indicated for growth inhibition assays, alkaline elution and drug transport.

RNase protection

The *mdr1* probe was a 174 bp *Bst*XI–*Acc*I fragment corresponding to nucleotides 1595–1768 of human *mdr1* cDNA.[15] Since the *mdr1* probe contains a 65 nucleotide region of uninterrupted sequence identity to *mdr1*[16] this probe can simultaneously detect *mdr1* and *mdr2* transcripts, yielding protected fragments of 174 and 65 nucleotides, respectively. A probe corresponding to a 210 bp *Ava*I fragment of the human *β-actin* cDNA was used as a ubiquitously expressed control. Additionally, each probe was allowed to hybridize with tRNA as a control for the effects of non-specific hybridization. The RNase protection procedure used was described previously.[17]

Results

The selection scheme for the VPR-2 subline shown in Figure 1 was modeled on common clinical usage of etoposide in combination therapy regimens for small cell lung cancer. In this setting the drug is given at 80–120 mg/M^2 on days 1–3 of a 21–28 day cycle. Peak serum levels in adults given a 100 mg/M^2 dose intravenously approximated 20 μM.[18] Thus, we exposed 10^8 H69 cells to etoposide 20 μM for 1 h daily for 3 days and repeated this approximately every 20 days. After the third treatment, a resistant population emerged and a cell line was established after serial passage *in vitro*. The drug resistance of VPR-2 cells has seen stable in the absence of drug exposure for 2.5 years.

Figure 2 demonstrates the 20-fold level of resistance of VPR-2 cells to growth inhibition by etoposide. This correlated well with resistance to etoposide-induced DNA strand breaking activity in whole cells (Figure 3). In isolated nuclei, however, the resistance was diminished to 3-fold because of increased sensitivity of VPR-2 and decreased sensitivity of H69 (Figure 3). Thus, although both

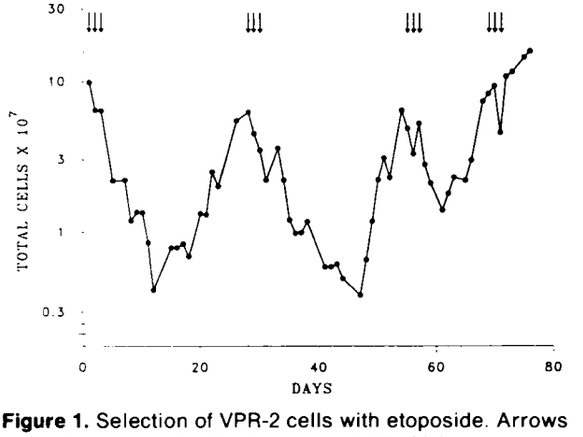

Figure 1. Selection of VPR-2 cells with etoposide. Arrows indicate treatment with etoposide at 20 μM for 1 h.

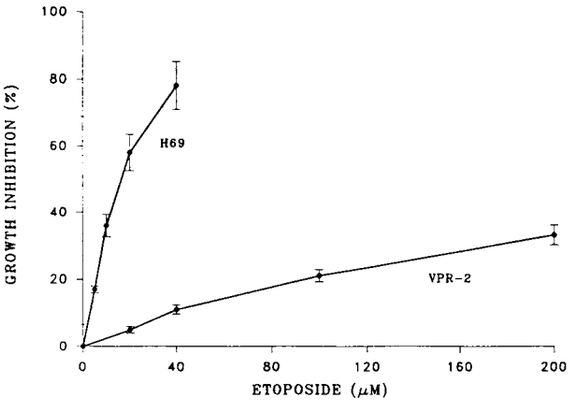

Figure 2. Etoposide-induced cytostasis using the MTT assay as described in Materials and methods. Values reflect means ± SD of at least three separate experiments.

cytoplasmic and nuclear factors contributed to the resistant phenotype, cytoplasmic factors accounted for the largest effect.

The cross-resistance profile of VPR-2 (Table 1) was consistent with 'classic' or 'typical' multidrug resistance associated with overexpression of P-glycoprotein and enhanced drug efflux, i.e. resistance to epipodophyllotoxins, intercalators and vinca alkaloids. However, steady-state concentrations of etoposide and daunomycin were reduced only 1.5- and 3-fold in the resistant line (Table 2). Although this nearly completely explained the level of resistance to daunomycin, it was insufficient to account for the degree of etoposide resistance in VPR-2.

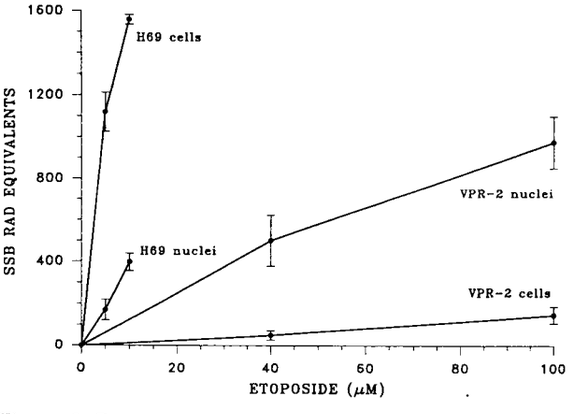

Figure 3. Etoposide-induced single-strand breaks (SSBs) measured in a low-sensitivity alkaline elution assay with proteinase K. Values reflect the means ± SD of three experiments.

Table 1. Dose modification factors VPR-2:H69[a]

Drug	Cytostasis[b]	DNA SSB activity[c]
Etoposide	20	24
Daunomycin	5.5	3
Mitoxanthrone	4	6
m-AMSA	2	3
X-radiation	1.2	1
Vincristine	20	NA[d]
Cisplatin	1	NA
5-Fluorouracil	1.5	NA

[a] Dose modification factor defined as factor by which drug dose must be multiplied to produce equitoxic effects in VPR-2 compared with H69.
[b] Assayed by MTT as described in Materials and methods.
[c] Single-strand breaks (SSBs) in DNA assayed by alkaline elution with proteinase K.
[d] Not applicable

The RNase protection assay demonstrated clear overexpression of the *mdr1* gene in VPR-2 cells relative to the parent line (Figure 4). By increasing the exposure time, a small amount of *mdr1* message was identified in H69 cells (data not shown). Notably, the *mdr2* gene, expressed in the human hepatoma cells (Figure 4, lane 3), is not expressed in either of the small cell lung cancer lines.

Based on the relatively small difference in etoposide concentration at the steady-state level and retention of some resistance to etoposide-induced protein-associated single-strand breaks in isolated nuclei, we examined topoisomerase II activity in crude nuclear extracts from both cell lines. However, no significant differences were identified in either catalytic activity, assayed by decatenation, or in etoposide-induced DNA cleavage assayed by generation of linearized SV40 DNA (form III) from form I substrate (data not shown).

In an effort to further characterize the mechanism of resistance to etoposide in VPR-2, we examined the effect of two resistance modifiers, verapamil and cyclosporin A. Both of these compounds are known to bind to P-glycoprotein; however, especially in the case of cyclosporin A, chemosensitizing activity is not always associated with this interaction and subsequent enhanced intracellular drug concentration. Verapamil at a suprapharmacologic dose (10 μM) was only modestly active (20- to 3-fold) as a chemosensitizer for etoposide and furthermore, there was no differential effect in the resistant subline (Table 3). Cyclosporin A in contrast produced a 7-fold sensitization ratio for etoposide in the resistant cells but was inactive in the parent line (Table 3 and Figure 5). Notably, in contrast to verapamil, the effect of cyclosporin A was seen

Table 2. Comparison of intracellular drug content in H69 and VPR-2 cells

Drug	Modifier	Concentration intracellular drug (nmol/g dry weight)[a]	
		H69	VPR-2
[^{3}H]etoposide (5 μM)	0	1.10 ± 0.03	0.70 ± 0.09
[^{3}H]etoposide (5 μM)	cyclosporin A (2 μg/ml)	1.27 ± 0.13	1.38 ± 0.18
[^{3}H]etoposide (5 μM)	verapamil (10 μM)	1.66 ± 0.05	0.99 ± 0.09
[^{3}H]daunomycin (2 μM)	0	0.59 ± 0.04	0.21 ± 0.02
[^{3}H]daunomycin (2 μM)	cyclosporin A (2 μg/ml)	0.76 ± 0.03	0.75 ± 0.03
[^{3}H]daunomycin (2 μM)	verapamil (10 μM)	0.65 ± 0.04	0.31 ± 0.02

[a] Values reflect two separate experiments with triplicate determinations ± SD.

at levels safely achieved in humans. Of interest, neither compound resulted in greater than 2-fold chemosensitization with daunomycin in either line.

Cyclosporin A similarly induced a marked enhancement of etoposide-induced strand-breaking activity in VPR-2 whole cells (Figure 6) but not in isolated nuclei (data not shown), indicating its effect occurred at the level of the cytoplasm, cytoplasmic membrane, or both. Similar experiments with the parent H69 line showed an enhancement of 1.5-fold or less in whole cells and no effect in isolated nuclei.

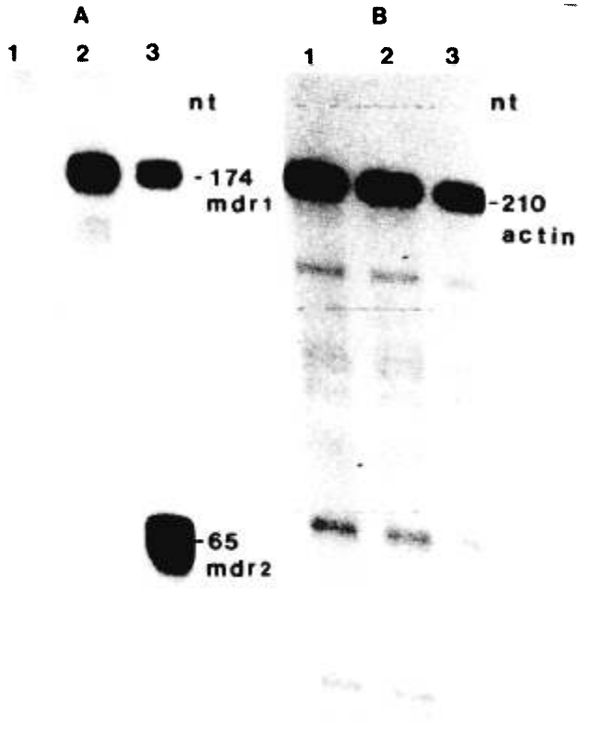

Figure 4. RNase protection assay with human *mdr1* (A) and *β-actin* (B) probes. Lane 1, H69 cells; lane 2, VPR-2 cells; lane 3, human hepatoma cells.

Cyclosporin A at 2 μg/ml completely normalized the steady-state concentration of etoposide in VPR-2 relative to H69 (Table 2). However, this did not explain its full effect. Total cellular content of [^{3}H]cyclosporin A was equivalent in both cell lines, suggesting that cyclosporin's effect was not mediated singularly through interaction with P-glycoprotein (Figure 7). In contrast to etoposide, the 2-fold

Table 3. Chemosensitization ratios for H69 and VPR-2 cells[a]

Cell line	Drug	Sensitization ratios[b]	
		cyclosporin A (2 μg/ml)	verapamil (10 μM)
H69	etoposide	1.2	2.1
	daunomycin	1.2	1.1
VPR-2	etoposide	7.4	3.2
	daunomycin	2.0	2.0

[a] Assayed by MTT.
[b] Sensitization ratio = ID_{50} without modifier/ID_{50} with modifier, ID_{50} is dose of drug reducing optical density to 50% of control.

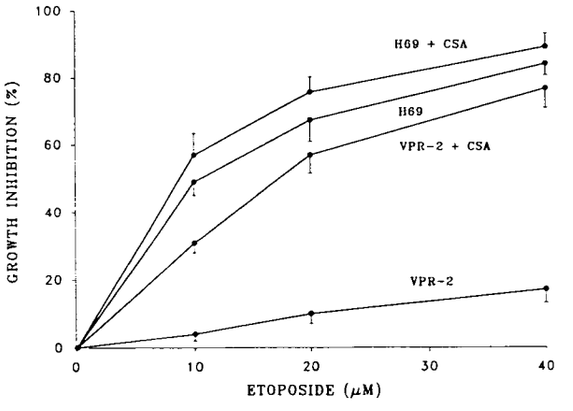

Figure 5. Effect of cyclosporin A (2 μg/ml) on etoposide-induced cytostasis using the MTT assay. Values reflect means ± SD from three separate experiments.

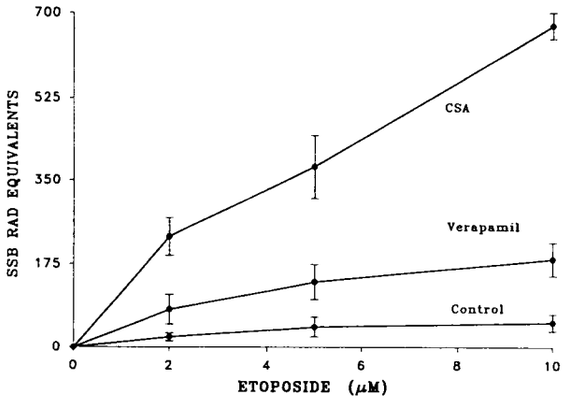

Figure 6. Effect of verapamil (10 μM) and cyclosporin A (2 μg/ml) on etoposide-induced single-strand breaks (SSBs) in VPR-2 cells measured by a high-sensitivity alkaline elution assay with proteinase K. Values reflect means ±SD from three separate experiments.

sensitization of daunomycin effect in VPR-2 cells by both verapamil and cyclosporin A is better correlated with enhanced intracellular concentrations of 1.5- and 3-fold, respectively.

Further characterization of cyclosporin's effect showed that simultaneous exposure to both agents was required and that chemosensitization was not enhanced by up to 2 h of pre-treatment with cyclosporin A. Minimal effects on cytostasis were seen at doses as low as 0.5 μg/ml, with a plateau in effect at levels above 2 μg/ml. The chemosensitizing effect of cyclosporin A in VPR-2 cells is unlikely to be mediated through an interaction with cyclophilin,

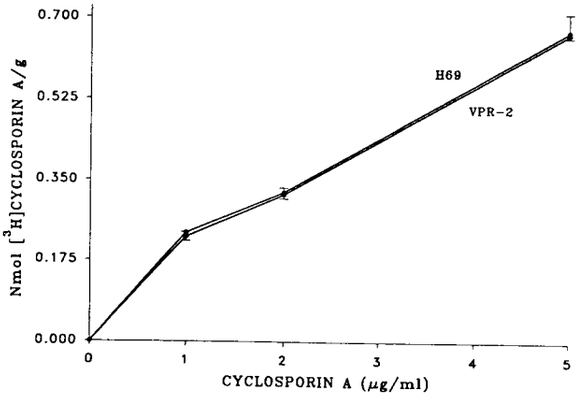

Figure 7. Total cellular content of [^{13}H]cyclosporin A expressed as nmole per gram dry cell weight. Values reflect means ±SD from triplicate samples.

the intracellular cyclosporin A binding protein critical for immunosuppressive activity, based on results with 11-methyl-leucine-cyclosporin A, a non-immunosuppressive analog that does not bind cyclophilin.[19] This analog did enhance etoposide effects in VPR-2 cells, but was approximately 2-fold less potent than cyclosporin A on a molar basis (data not shown), as previously reported in other cell lines.[20] KF506, an immunosuppressive agent which binds to a different intracellular protein that does cyclosporin A, was devoid of chemosensitizing activity for etoposide in both VPR-2 cells and the parent line.

Discussion

The rapid selection of the VPR-2 cell line and the stability of the multidrug-resistant phenotype suggest that these cells may have been a steady subpopulation of the H69 parent line through years of serial passage *in vitro*. It is conceivable that the small amount of *mdr1* gene product detected in total RNA from H69 cells reflects the subpopulation that is VPR-2. It may be possible to prove this by flow sorting H69 cells after binding of labeled antibody to a cell surface epitope of P-glycoprotein and comparing the sorted population with VPR-2 cells.

Although VPR-2 cells overexpressed P-glycoprotein relative to the parent line, our data indicate that additional factors contributed to etoposide resistance in these cells. The strongest evidence for this was the differential sensitization, relative to the parent cells, of VPR-2 cells to etoposide produced by simultaneous treatment with cyclosporin A only and not verapamil. This occurred despite similar effects of the two compounds on intracellular etoposide concentration (2- and 1.4-fold enhancement, respectively). Furthermore, despite normalization of intracellular etoposide and daunomycin concentrations by both cyclosporin A and verapamil, resistance was not completely reversed in VPR-2 cells. The differential effect of cyclosporin A on etoposide sensitivity in VPR-2 cells may offer a clue to an additional mechanism of resistance operative in this line.

Although both verapamil and cyclosporin A are known to bind to P-glycoprotein,[21–24] several lines of evidence suggest that these agents probably enhance chemosensitivity through multiple pathways (for a review see Twentyman[25]). Cyclosporin A possesses broad chemosensitizing activity in many parental 'sensitive' cell lines that do not have

detectable P-glycoprotein expression. Further, this effect in both parent and resistant sublines is only inconsistently associated with increased intracellular drug accumulation, inhibition of drug efflux or both. In this vein, we have tested SDZ PSC 833, a cyclosporin analog previously reported as 5- to 10-fold more active than cyclosporin A at reversing resistance, presumably on the basis of higher affinity for P-glycoprotein.[26] However, specifically in VPR-2 cells treated with etoposide, PSC 833 was only 2-fold more effective than cyclosporin A as a chemosensitizer. Notably, this difference was observed only at concentrations below 1 μM and was not consistently associated with comparable increases in steady-state etoposide levels (unpublished data).

It has been hypothesized that cyclosporin A may exert its chemosensitizing effects in part through its effects on the biophysical properties of membranes. Cyclosporin A is known to partition into phospholipid vesicles and disrupt membrane architecture, as well as depolarize membrane potentials.[27–29] Changes in the lipid composition of membranes from some multidrug-resistant cell lines have been reported and changes in membrane fluidity possibly associated with this chemical change have also been noted.[30–32] Cyclosporin A has been shown to restore the resting membrane potential of resistant human and murine leukemia cell lines to that of the sensitive parent cells.[33]

Intracellular effects associated with chemosensitizing activity in addition to binding to P-glycoprotein have also been noted for verapamil. Most notably, verapamil is known to correct the altered subcellular distribution of anthracyclines observed in resistant cells, presumably resulting in greater access of drug to critical intracellular targets such as DNA. In the first study to document this effect, fluorescent microscopy showed a punctate cytoplasmic distribution of daunorubicin in resistant HL-60/AR cells, which do not express P-glycoprotein, compared with a homogeneous pattern in the parent line.[34] Verapamil's ability to correct this altered pattern of drug distribution was correlated with its ability to effect movement of daunorubicin from a lipophilic to a hydrophilic compartment in an *in vitro* system. Several other resistance modifiers were also active in this regard; however, notably, extremely high concentrations of cyclosporin A were required for this effect compared with those of verapamil, trifluoperazine and tamoxifen. Other investigators have also identified altered subcellular distribution of anthracyclines in P-glycoprotein positive-lines and correction of this with verapamil.[35,36]

Conclusion

It is certainly conceivable that altered subcellular distribution plays a role in the drug resistance displayed by VPR-2 cells. However, unlike previous cell lines in which this has been studied, the major interaction in VPR-2 cells would not be predicted to be verapamil and daunomycin, in which case chemosensitization seems to be completely explained by enhanced intracellular drug accumulation. Instead, given the discrepancy between drug accumulation and chemosensitizing effects, and given the correlation with enhanced drug-induced strand-breaking activity in whole cells but not nuclei, we would hypothesize that VPR-2 cells sequester etoposide in the cytoplasm and that cyclosporin A acts to free this sequestered drug, facilitating access to critical targets in the nucleus. To investigate this, we are currently developing methodology using radiolabeled etoposide and cellular fractionation techniques to characterize drug distribution.

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