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Correction of altered plasma membrane potentials

A possible mechanism of cyclosporin A and verapamil reversal of pleiotropic drug resistance in neoplasia

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Summary. We have recently shown that cyclosporin A (CsA) reverses pleiotropic drug resistance in human acute lymphatic leukemia in vitro and daunorubicin resistance in Ehlrich ascites tumor in vivo. In the present study we examined the mechanisms by which CsA might reverse pleiotropic drug resistance relative to changes in cellular plasma membrane potentials and intracellular calcium ($[Ca^{2+}]_i$). Membrane potentials were measured with DIOC5 dye flowcytometrically and $[Ca^{2+}]_i$ levels with Quin 2 dye spectrofluorimetrically. All pleiotropic (PDR) drug-resistant tumor sublines had decreased membrane potentials (membrane depolarized) compared with their corresponding drug-sensitive parental tumors. In comparison, the membrane potentials of a control antimetabolite-resistant acute leukemia cell line were unchanged. The basal levels of $[Ca^{2+}]_i$ in the PDR sublines were variable compared with those of parental drug-sensitive cell lines. Incubation of all PDR tumor sublines with CsA or verapamil resulted in the restoration of membrane potentials to that characteristic of the corresponding drug-sensitive parental tumor. Cyclosporin A produced variable changes in the levels of $[Ca^{2+}]_i$. These data suggest that alteration of membrane potentials is one of the mechanisms responsible for pleiotropic drug resistance in malignancy and show that this alteration is corrected by CsA and verapamil.

Introduction

The development of drug resistance by malignant cells is an important and challenging area of cancer treatment research. During the past 5 years rapid progress has been made toward understanding the phenomenon of pleiotropic drug resistance, by which neoplastic cells exposed to a single chemotherapy drug also develop resistance to multiple, unrelated chemotherapeutic agents [11]. The search for mechanisms has focused at the level of tumor cell membrane, cytoplasm, DNA repair, and gene amplification [11]. However, there is no agreement on any one mechanism responsible for pleiotropic drug resistance (PDR); perhaps multiple mechanisms are responsible. Several investigators have reported that verapamil hydrochloride, a calcium channel blocker, reverses PDR in vitro [10, 17, 18, 23–25]. Recently we have shown that cyclosporin A (CsA) reverses multiple drug resistance in vitro and in vivo [19, 20]. Because CsA has been shown to change membrane potentials in murine lymphocytes and influence calcium-calmodulin pathways [3, 4], we compared the membrane potentials and intracellular calcium ($[Ca^{2+}]_i$) levels in resistant and sensitive tumor cell lines and examined the in vitro effects of CsA and verapamil on these two parameters.

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Materials and methods

Cell lines

Four drug-resistant tumor sublines and their corresponding parental drug-sensitive tumors were used for the present study.

Human acute lymphatic leukemia. We developed a pleiotropic drug-resistant subline of human T cell ALL, GM 3639, Human Genetic Mutant Cell Repository, Camden, NJ, by continuous in vitro exposure of drug-sensitive parental cells to increasing concentrations of vincristine [18]. The resistant subline shows primary resistance to vincristine (VCR) and cross-resistance to daunorubicin (DNR) and to VP-16 [18]. The parental cell line is designated L_0 and the resistant subline, L_{100}. A methotrexate-resistant subline of GM 3639 cells was developed by continuous exposure of parental cells to initially sublethal (3 nmol) and progressively increased concentrations of methotrexate (MTX). The MTX ED_{50} for a 50% kill of GM 3639 cells in a 3-day assay is 4.3 ±1.5 nmol. The resistant subline is 7-fold resistant, grown continuously in 30 nmol MTX, and designated L_{30} MTX.

Murine P388 leukemia. Adriamycin-sensitive (P388) and an adriamycin-resistant subline of P388 (P388/ADR) were obtained from the DTC Tumor Repository, Division of Cancer Treatment, National Cancer Institute, NSC-123127. P388 leukemia was originally induced by methylcholanthrene in a DBA/2 mouse [6]. The P388/ADR cell line is cross-resistant to DNR, VP-16, and VCR.

In vitro, 2.57 ±1.24 μg/ml DNR is required to inhibit 50% of DNA synthesis (DNR ED_{50}) in a 1-h DNR incubation assay in P388 cells versus 34.7 ±3.8 μg/ml DNR in P388/ADR cells; 67.0 ±46.2 nmol VCR (VCR ED_{50}) and 82.5 ±10.6 nmol VP-16 are required to produce a 50% kill of P388 cells in a 3-day viability assay as opposed to 2333 ±252 nmol VCR and >10,000 nmol VP-16 in P388/ADR

cells. The DNR ED_{50} of P388/ADR is reduced to 4.7 ±3.2 μg/ml and the VCR ED_{50} of these cells is reduced to 62.5 ±30.4 m*M* by 5.5 μg/ml CsA.

In vivo, 1×10^6 P388 cells produce death in nontreated control mice in 9.8 ±1.1 days compared with 10.2 ±0.8 days in mice inoculated with P388/ADR cells; DNR 2.4 mg/kg i.p. on days 1, 3, and 5 produces a mean survival time (MST) of 18.9 ±2.7 days in mice bearing P388 vs 11.5 ±0.9 days in mice bearing P388/ADR; VP-16 10 mg/kg on days 1 and 3 produces an MST of 27.8 ±3.7 days in mice bearing P388 as opposed to 13.8 ±1.9 days in mice bearing P388/ADR, and VCR 1.5 mg/kg on day 3 produces an MST of 19.1 ±2.3 days in mice bearing P388 vs 10.1 ±1.3 days in mice bearing P388/ADR ($P < 0.05$ for all treatment comparisons; unpublished observations).

Ehrlich ascites carcinoma. Ehrlich ascites carcinoma was obtained as a gift from Dr. Steven Armentrout, University of California, Irvine. We developed a DNR-resistant subline of this tumor by transfering Ehrlich ascites carcinoma cells to 18 sequential generations of host BALB/C mice with continuous DNR treatment. The sensitive cell line is designated as EA/DS and the resistant subline, EA/DR [17].

Hepatoma-129. Murine hepatoma (H-129) was obtained from the DTC tumor repository at NCI Frederick Cancer Research Facility, Frederick, Md. This tumor grows as a malignant ascites in C3H/HEN mice and produces a nontreated MST of 21.9 ±0.9 days after i.p. inoculation of 0.2 ml malignant ascites harvested at 1 week. A DNR-resistant subline (H-129/DNR) was developed by continuous treatment of 9 sequential transfer generations of host mice bearing H-129 with DNR, 0.4 mg/kg on days 1 and 2 after tumor inoculation. The MST of host mice bearing H-129/DNR treated with DNR alone is 22.2 ±2.5 days compared with 29.9 ±3.6 days when treated with combined CsA (10 mg/kg) and DNR ($P < 0.001$). This same DNR regimen produces an MST of 27.5 ±4.7 days in mice bearing the drug-sensitive parental hepatoma, $P < 0.01$ vs nontreated control (unpublished observations).

Reagents

3,3′-Dipentyloxacarbocyanine iodide (DIOC5) and Quin 2 acetoxymethylester were purchased from Molecular Probes, Junction City, Ore.

Measurements of membrane potential

Tumor cells were washed in Hanks' balanced salt solution (HBSS) and resuspended in RPMI-1640 containing 5% fetal calf serum (Irvine Scientific, Irvine, Calif.) at 5×10^6/ml. Cell suspensions (100 μl) were aliquoted into 12 × 75 mm polypropylene tubes. Cyclosporin A (6.6 μg/ml), verapamil (5 μg/ml), or daunorubicin (4 μg/ml) were added to corresponding tubes (concentrations of cyclosporin A and verapamil were used based on their in vitro capacity to correct pleiotropic drug resistance as measured by cytotoxicity assay, and for daunorubicin the concentration used was similar to that producing over 50% inhibition of DNA synthesis as measured by inhibition of thymidine incorporation in sensitive tumor cells) and incubated at 37°C for 5 min. A 5 μ*M* solution of DIOC5 was prepared in phosphate-buffered saline and prewarmed at 37°C. Of this solution, 0.9 ml was added to the cell suspension (with or without drugs), immediately after which cell membrane potentials were recorded by flowcytometry using a FACS analyzer according to the technique of Monroe and Cambier [12] with minor modifications. Data were recorded on a log scale. Resting membrane potentials from sensitive cell lines were adjusted on the fluorescence scale by adjusting the FL 1-PMTs on a FACS analyzer (Becton-Dickinson, Mountainview, Calif). Cells were gated on volume vs scatter for uniform size distribution. Membrane was depolarized when fluorescence intensity decreased (lower mean channel numbers than in control) and hyperpolarized when fluorescence intensity increased (higher mean channel numbers than in control). Membrane potential measurements were done simultaneously, under the same experimental conditions in drug-resistant and as in the corresponding parental drug-sensitive cell lines, and compared.

Measurements of intracellular calcium

Cells (10×10^6) in 1 ml medium were loaded with 80 μ*M* Quin-2 for 45 min at 37°C, wahed twice with HBBS, and then resuspended in a saline solution containing 145 m*M* NaCl, 1 m*M* Na_2HPO_4, 0.5 m*M* $MgSO_4$, 5 m*M* KCl, 1 m*M* $CaCl_2$, 5 m*M* glucose, and 10 m*M* HEPES (pH 7.4). Cells (5×10^6) in saline solution were placed in a temperature-controlled (37°C) quartz cuvette for each experiment, and measurements were made with a spectrofluorometer (SPEX, Fluorog, Metuchen, NJ), with excitation at 339 nm and emission at 484 nm. Spectrofluorometer output was recorded on FM tape and was digitized off-line with a LSI-11/23 computer with data interface. Fluorescence intensity was converted to free Ca^{2+} concentration as has previously been described [22].

Results

Plasma membrane potential

Membrane potentials of drug-sensitive and -resistant cell lines were recorded after 5 min exposure to CsA, verapamil, DNR, or no drugs. The results for resistant (L_{100}) and sensitive (L_0) cell lines are shown in Figs. 1 and 2, respectively. The membrane potentials of PDR-resistant L_{100} cells (Figs. 1) were markedly decreased (mean channel numbers 14.5; membrane depolarized) compared with those of sensitive L_0 cells (mean channel numbers 43.9; Fig. 2). CsA and DNR decreased the membrane potentials of L_0 cells, whereas verapamil had no effect. In contrast, CsA shifted the membrane potentials (mean channel numbers 34.2) in L_{100} cells to the range of control L_0 cells (mean channel numbers 43.9). Verapamil had a less striking effect (mean channel numbers 25.4) and DNR had no effect (mean channel numbers 14.3) in correcting the membrane potential of L_{100} cells.

Membrane potential analysis of resistant P388/ADR (P388/R) leukemia showed two peaks of fluorescence representing two types of cells (Fig. 3): a smaller population with membrane potentials (mean channel numbers 28.4) comparable to those of the parental, sensitive P388 P388/S, mean channel numbers 29.5; Fig. 4), and a second

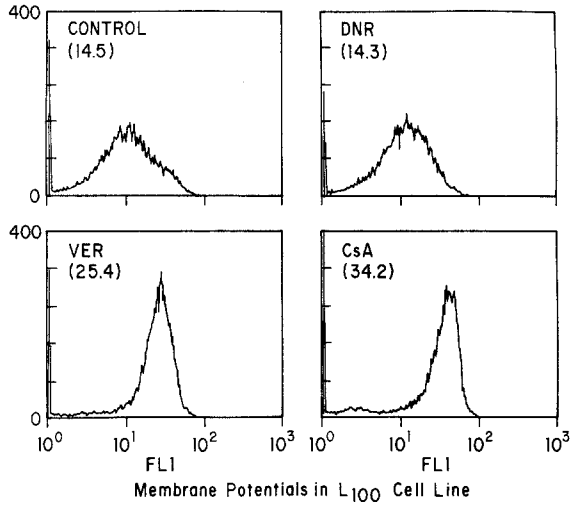

Fig. 1. Membrane potentials in L_{100} (resistant cells). Data in parentheses are shown as mean log fluorescence channels for control cells and following exposure to daunorubicin (*DNR*), verapamil (*Ver*), and cyclosporin A (*CsA*)

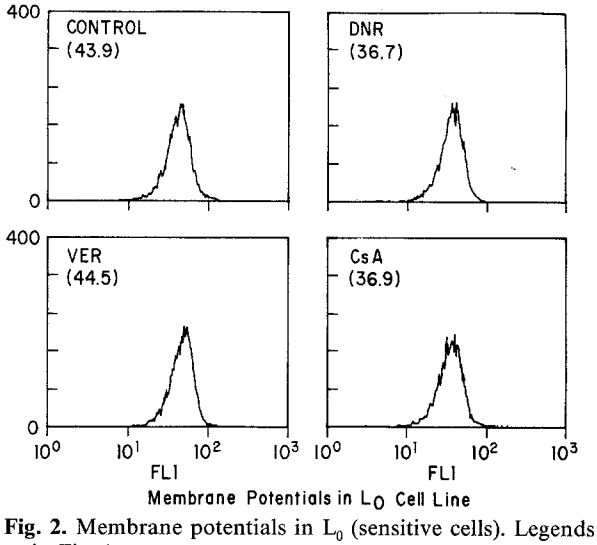

Fig. 2. Membrane potentials in L_0 (sensitive cells). Legends same as in Fig. 1

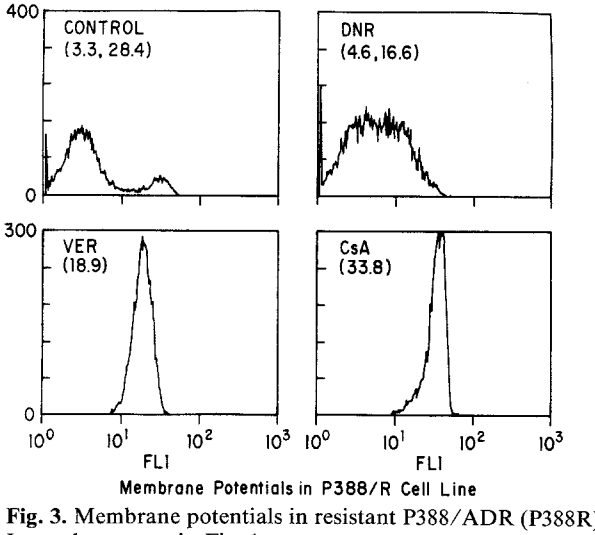

Fig. 3. Membrane potentials in resistant P388/ADR (P388R) cells. Legends same as in Fig. 1

larger population in which cell membranes were markedly depolarized (mean channel numbers 3.3). Cyclosporin A completely corrected the membrane potential fluorescence peaks of P388/ADR cells, resulting in a single peak of fluorescence (mean channel numbers 33.8) representing a single population of cells with membrane potentials similar to those of the drug-sensitive parental cell line. Although verapamil produced a single peak of fluorescence representing a single population of cells, it only partially restored the membrane potentials of P388/ADR cells (mean channel numbers 18.9) compared with those of the parental cell line. In contrast, DNR had no effect on the membrane potential of the larger population of P388/ADR cells with markedly depolarized potentials (mean channel numbers 3.3 in control vs 4.6 with DNR), whereas DNR induced depolarization of the smaller population of P388/ADR cells with plasma membrane potentials similar to those of the sensitive P388S cells (mean channel numbers 28.4 in control vs 16.6 with daunorubicin) and two cell populations persisted. In P388 cells, CsA and DNR produced depolarization, whereas verapamil had no effect.

The membrane potential analysis of Ehrlich ascites carcinoma was similar to that of the other two cell lines. The membrane potentials of EA/DR cells were depolarized (mean channel numbers 20.7; Fig. 5) compared with those of EA/DS cells (mean channel numbers 36.2; Fig. 6). Both CsA and verapamil partially corrected the membrane potentials of EA/DR cells (mean channel numbers 27.0 and 24.8, respectively), whereas daunorubicin had no effect on EA/DR cells (mean channel numbers 20.4). In EA/DS cells, CsA and verapamil had a minimal effect on membrane potentials; however, DNR induced depolarization.

Results of the membrane potential analysis of resistant and sensitive murine hepatocellular carcinoma are shown in Figs. 7 and 8, respectively. Membrane potentials were decreased (mean channel number 25.1) in H-129/DNR (Fig. 7) compared with the sensitive H-129 cell line (mean channel number 32.7) (Fig. 8). Both CsA (mean channel number 28.0) and DNR (mean channel number 29.2) depolarized plasma membranes in sensitive H-129 cells. Daunorubicin had no significant effect on the membrane

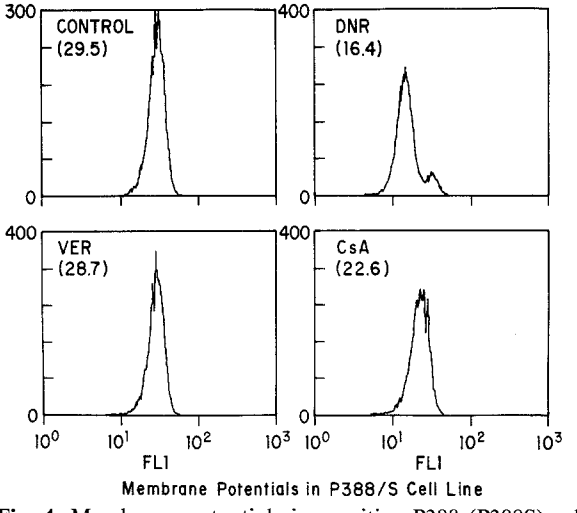

Fig. 4. Membrane potentials in sensitive P388 (P388S) cells. Legends same as in Fig. 1

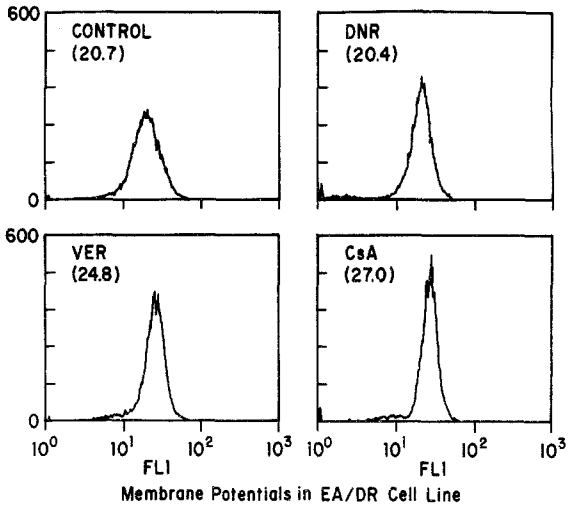

Fig. 5. Membrane potentials in resistant EA/DR cells. Legends same as in Fig. 1

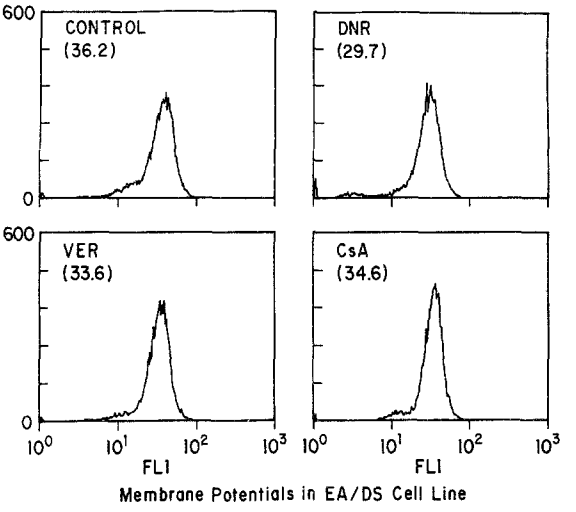

Fig. 6. Membrane potentials in sensitive EA/DS cells. Legends same as in Fig. 1

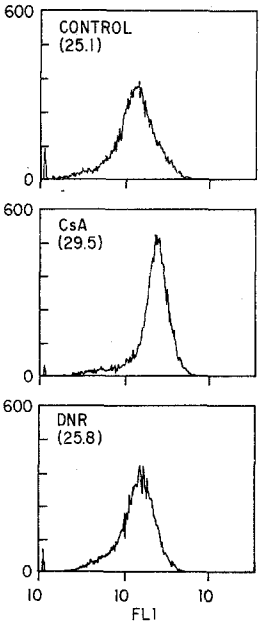

Fig. 7. Membrane potentials in resistant H-129 (H-129/ADR) cells. Legends same as in Fig. 1

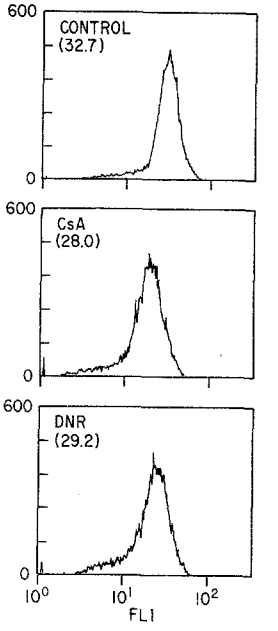

Fig. 8. Membrane potentials in sensitive H-129 cells. Legends same as in Fig. 1

potentials of H-129/DNR cells (mean channel number 25.8), whereas CsA repolarized the plasma membrane of resistant H-129/DNR cells to the range of sensitive H-129 cells (mean channel number 29.5). In contrast, the membrane potentials of 7-fold MTX-resistant GM 3639 were essentially the same as those of the parental drug-sensitive tumors, 22.5 $\pm$1.2 vs 24.5 $\pm$0.9, respectively ($P > .01$) (data not shown).

Data were highly reproducible and statistically significant. An example is shown in Table 1, which presents the effect of verapamil and CsA on the membrane potentials of L_{100} and H-129/DNR cells.

Intracellular calcium

The results of $[Ca^{2+}]_i$ analysis in PDR and sensitive cell lines are summarized in Table 2. The basal level of $[Ca^{2+}]_i$ in the resistant cells compared with that in the parental, sensitive cells were either increased (in L_{100} cells compared with L_0 cells) or decreased (in P388/ADR and H-129/DNR compared with P388 and H-129). Cyclosporin A had little effect on $[Ca^{2+}]_i$ in P388 cells, whereas DNR decreased the basal level of $[Ca^{2+}]_i$ in both P388/ADR and P388 cells. CsA increased $[CA^{2+}]_i$ in both L_0 and L_{100} cells, whereas DNR decreased $[Ca^{2+}]_i$ in L_{100} cells and had almost no effect in L_O cells. Verapamil had no effect on $[Ca^{2+}]_i$ in L_0 and L_{100} cells. Cyclosporin A increased $[Ca^{2+}]_i$ levels in both sensitive and resistant hepatoma. In contrast, DNR decreased $[Ca^{2+}]_i$ in both resistant and sensitive cells. These data would indicate a lack of consistent effect of CsA and verapamil on basal $[Ca^{2+}]_i$ levels in the tumor cell lines studied.

All the experiments were repeated at least twice on each cell line and the results were reproducible within $\pm$10 nmol.

Discussion

In the present study we have shown that the plasma membranes of drug-resistant sublines of acute lymphatic leuke-

Table 1. Plasma membrane potentials in PDR cell lines[a]

Cell lines	Membrane potentials (mean channel numbers)	*P* value
[a] L_0	31.4 ± 2.0	–
[b] L_{100}	19.8 ± 2.0	< 0.001 vs [a]
[c] L_{100} + CsA[b]	30.9 ± 2.9	> 0.1 vs [a]; < 0.01 vs [b]
[d] L_{100} + Ver[b]	26.3 ± 1.8	> 0.05 vs [a]; < 0.02 vs [b]
[a] H-129	27.5 ± 0.6	–
[b] H-129/DNR	19.3 ± 1.0	< 0.001 vs [a]
[c] H-129/DNR + CsA[b]	25.5 ± 0.8	> 0.05 vs [a]; < 0.01 vs [b]
[d] H-129/DNR + Ver[b]	22.9 ± 0.3	< 0.02 vs [a] and [b]

[a] Membrane potentials shown in this table are different from those shown in the figures for respective tumors because of differences in PMTs used on FACS analyzer during measurements
[b] Cyclosporin 0.1 μ*M* and verapamil 0.1 μ*M*

Table 2. Intracellular calcium in sensitive and resistant tumor cells

Agents	$[Ca^2]_i$ in nmol					
	Sensitive L_0	Resistant L_{100}	Sensitive P388	Resistant P388/ADR	Sensitive H-129	Resistant H-129/DNR
None	73.1	102.9	80.2	55.1	146.4	121.0
CsA	106.5	126.9	89.6	49.9	204.4	292.7
Verapamil	73.1	102.9	ND	ND	ND	ND
DNR	66.2	64.0	39.9	42.8	59.2	45.2

ND, Not done; DNR, daunorubicin; CsA, cyclosporin A

mias, murine hepatoma, and Ehrlich ascites carcinoma are markedly depolarized compared with those of their respective parental drug-sensitive tumors. Previous evidence has shown that anthracycline antibiotics are active against tumor cell plasma membranes since these drugs are cytotoxic to cultured L 1210 leukemia and drug-resistant hepatocytes when bound to insoluble carriers, which prevent intracellular drug entry [15, 21]. Our study shows that DNR depolarizes the plasma membranes of drug-sensitive acute leukemias, hepatoma, and Ehrlich ascites carcinoma, whereas it has little effect on the previously depolarized membranes of the drug-resistant sublines derived from these tumors. It is interesting to note that DNR had no effect on the larger population of abnormally depolarized P388/ADR cells, but, similar to its effect on sensitive P388 cells, depolarized the smaller population of P388/ADR cells. It has recently been shown that plasma membrane ion leakage and the loss of membrane potentials are early events in the cytotoxic effects of hemolytic paramyxoviruses and that other membrane-directed cytotoxic agents share similar actions [1, 26]. It is probable that anthracycline cytotoxicity mediated at the level of plasma membrane involves plasma membrane depolarization. It would follow that subpopulations of partially depolarized cells in a heterogenous parental population might be selected upon continuous exposure to DNR, resulting in membrane-depolarized, resistant subclones.

Davis et al. [5] have recently shown that membrane potentials influence drug transport in neoplastic cells. MCF-7, a human breast adenocarcinoma cell line, has elevated mitochondrial and plasma membrane potentials compared with normal epithelial cells. The former cell line shows a greater uptake and retention of lipophilic cationic compounds but can be made to resemble normal cells in the transport and retention of these agents by dissipation of its hyperpolarized mitochondrial membrane potential [5]. In recent years, calcium channel blocking agents have been shown to enhance the response of drug-resistant tumor cells to vinca alkaloids and anthracycline antibiotics. The mechanism of this effect seems to be due in part to inhibition of the enhanced efflux of these drugs by resistant tumor cells, resulting in increased intracellular drug retention [23, 24]. However, since the acquisition of equimolar concentrations of anthracyclines by pleiotropic drug-resistant tumor cells compared with drug-sensitive tumor cells fails to restore equivalent cytotoxicity, the mechanism(s) of restoration of drug sensitivity must extend beyond drug retention [8, 10, 14, 16].We have recently shown that CsA reverses multidrug resistance in human acute lymphatic leukemia cells in vitro and DNR resistance in DNR-resistant Ehrlich ascites carcinoma cells in vitro and in vivo [19, 20]. We failed to find CsA-induced alteration of initial or early DNR transport in the acute lymphatic leukemia cell line, although Koletsky et al. have recently noted enhanced DNR uptake by CsA in this cell line over a prolonged period of drug exposure [A. Koletsky, personal communication].

In the present investigation we studied other mechanisms by which CsA might reverse multidrug resistance. Because CsA acts in the calcium pathway and depolarizes the membranes of murine lymphocytes [3, 4], we compared these parameters between sensitive and resistant tumor cells and examined the effect of CsA and verapamil on membrane potentials and $[Ca^{2+}]_i$. We observed that CsA repolarized the membranes of drug-resistant tumor sublines and restored their membrane potentials to those char-

acteristic of drug-sensitive parental tumors. Although to a lesser extent, verapamil had similar effects. These data suggest that the depolarization of membranes might be one of the mechanisms of PDR and that CsA and verapamil reverse resistance by the correction of altered membrane potentials. Membrane depolarization was observed in all four PDR cell lines. Membrane hypopolarization has recently been observed by Hasmann et al. [9] in adriamycin-resistant Friend leukemia and is probably unique to PDR since the membrane potentials of our MTX-resistant ALL subline remain unaltered. Our experiments failed to show any consistent alteration in $[Ca^{2+}]_i$ concentration in resistant or sensitive tumor cells or any consistent CsA effect on $[Ca^{2+}]_i$.

Since anthracycline-resistant P388 cells have the same calmodulin content as sensitive cells but respond selectively to CsA, it is unlikely that CsA reverses drug resistance by inhibition of the calmodulin-dependent activation of phosphodiesterase [2, 3, 13]. It is also unlikely that CsA reverses drug resistance by binding to cyclophilin, a specific CsA-binding cytoplasmic protein, since Koletsky has shown that cyclophilin levels in our drug-sensitive and PDR GM 3639 cells are essentially the same [7; A. Koletsky, personal communication].

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