

MEMBRANE POTENTIAL DIFFERENCES BETWEEN ADRIAMYCIN-SENSITIVE AND -RESISTANT CELLS AS MEASURED BY FLOW CYTOMETRY*

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Abstract—Using the fluorescent membrane potential probe, 3,3'-dihexyl-oxacarbocyanine (DiOC6(3)), we found a 4-fold higher uptake in Adriamycin (ADM)-sensitive versus -resistant Friend leukemia cells (FLC). When sensitive cells were treated in the presence of high potassium (120 mM K⁺), there was a greater than 80% reduction of DiOC6(3) uptake. Using carbonylcyanide 4-trifluoromethoxyphenylhydrazone (FCCP), a specific inhibitor of mitochondrial membrane potential, DiOC6(3) accumulation was reduced by less than 30% in these cells. Both results support the conclusion that a greater uptake of DiOC6(3) in ADM-sensitive than in -resistant cells indicates an increased plasma transmembrane potential. Since electronegative plasma membrane potentials are a driving force for the transport of lipophilic positively-charged compounds, differences in membrane potentials between sensitive and multiple drug resistant (MDR) tumor cells could have an important influence on drug accumulation and cytotoxicity. The drugs which our ADM-resistant FLC display multiple drug resistance to are positively charged. In MDR FLC, the calcium channel antagonist, verapamil, has been shown to block the efflux of Rhodamine 123 (Rho 123) and other positively-charged compounds. Since DiOC6(3) is also positively-charged, we used verapamil to investigate its effects on drug uptake. In MDR FLC, verapamil increased DiOC6(3) accumulation by 1.9-fold, whereas in sensitive cells it was increased 1.5-fold. In contrast, verapamil increased the levels of Rho 123 in resistant cells 7.8-fold but lowered them in sensitive cells 1.5-fold. The minimal loss of DiOC6(3) from both sensitive and MDR cells and the above results can best be interpreted as indicating that DiOC6(3) is not transported by the efflux "pump" system but that verapamil induces a plasma membrane potential increase in sensitive and resistant cells that DiOC6(3) is sensitive to. On the other hand, since Rho 123 did appear to be actively effluxed from these resistant cells, the enhancement of this compound by verapamil was more likely due to inhibition of the MDR "pump." How, or whether, plasma membrane potentials and the MDR efflux "pump" are related remains to be investigated. In the resistant cells, verapamil also induced an increase (13-fold) in the accumulation of the electrically neutral fluorescent probe for calcium, INDO-1/AM. However, verapamil had no effect on the efflux of this compound, which was equivalent in both resistant and sensitive cells. Thus, a new effect of verapamil on drug accumulation in MDR cells is identified here.

We have shown previously that a number of positively-charged fluorescent dyes, such as Rhodamine 123 (Rho 123), Rhodamine 6G, and Saffranin O, preferentially accumulate in, and are more cytotoxic to, a human breast carcinoma cell line, MCF-7, as compared to an epithelial cell line derived from normal tissue, CV-1 [1]. More recently, similar results have been obtained in these cells with the positively-charged anticancer agent, Adriamycin (ADM) [2]. Using microelectrodes, we found that these increases in drug accumulation correlate with increased transmembrane potentials in MCF-7 vs CV-1 cells [1]. These results are consistent with the

interpretation that electrically negative membrane potentials influence the accumulation of positively-charged compounds.

However, it has been widely reported that decreased accumulation of ADM and its analogs is related to the ability of a tumor cell to efflux these and a variety of other seemingly unrelated compounds [3-9]. Dano [4], Skovsgaard [5] and other investigators [6] have shown, by use of lowered glucose and metabolic inhibitors, that drug efflux is an active process, and they proposed the existence of an efflux "pump" to explain this phenomenon [7]. The acquired or inherent ability of a tumor cell to efflux a number of seemingly unrelated drugs has been linked to resistance to these agents; hence, the term multiple drug resistance (MDR) has emerged. Other mechanisms and biochemical changes which could contribute to cellular resistance for these numerous compounds have been reported, including: increased glutathione levels [10], increased DNA repair [11, 12], membrane fluidity differences

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[13,14], cytogenetic changes [15], and increased intracellular calcium [16].

Treating Friend leukemia cells (FLC) with stepwise increases in ADM concentrations, we have derived variants with different degrees of MDR [15]. These resistant variants also have increased efflux and reduced accumulation of the positively-charged compounds, ADM and Rho 123 [7]. Therefore, with flow cytometry, we investigated the transmembrane potentials of these sensitive and MDR resistant FLC using the positively-charged membrane potential fluorescent probe 3,3'-dihexyl-oxacarbocyanine (DiOC6(3)). Since elevated calcium levels have been shown to occur in drug-resistant cells [16], we also measured, by flow cytometry, cytoplasmic calcium in our resistant and sensitive cell lines, using the calcium fluorescent probe INDO-1/AM in the presence and absence of the calcium channel blocker verapamil.

MATERIALS AND METHODS

Reagents and preparation of stocks. Propidium iodide (PI), Rho 123 and fluorescein isothiocyanate (FITC) were purchased from Sigma, Deisenhofen, F.R.G.; 3,3'-dihexyl-oxacarbocyanine iodide (DiOC6(3)) was from Eastman Kodak Co., Rochester, NY, U.S.A. Indo-1-pentaacetoxymethyl ester (INDO-1) was obtained from Calbiochem, Frankfurt a.M., F.R.G.; carbonylcyanide 4-trifluoromethoxyphenylhydrazone (FCCP) from Aldrich, Steinheim, F.R.G.; verapamil from Searle, Chicago, IL, U.S.A.; and A23187Br from the HSC Research Development Corp., Toronto, Canada.

Stock solutions of PI (3 mM), DiOC6(3) (17.5 μ M), Rho 123 (1.3 mM), INDO-1 (1 mM), FCCP (1 mM), and A23187Br (1.65 mM) were prepared in dimethylformamide (Baker, Deventer, The Netherlands); verapamil (1 mM) was prepared in 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline, pH 7.35 (HBS), CaCl₂ (0.05 M) and EDTA (0.1 M). FITC was conjugated to monodispersed and porous latex particles with free amino groups and a 5 μ m diameter (Paesel, Frankfurt a.M., F.R.G.) by incubation of 2×10^8 particles/ml for 2 hr at pH 9.5 in a 50 mM NaHCO₃ buffer with 0.25 mg/ml FITC followed by three washing steps with 50 ml HBS. The final suspension in HBS was adjusted to a particle concentration of 5×10^6 particles/ml.

Cell lines and culture conditions. Multiple drug-resistant cells (ADM-RFLC3) were derived from drug-sensitive Friend virus transformed murine leukemia cells (FLC) as described earlier [15].

Both cell lines were seeded in Dulbecco's modified Eagle's medium (Gibco, Eggenstein, F.R.G.) supplemented with 10% fetal calf serum (Gibco) plus 100 units/ml penicillin and 100 μ g/ml streptomycin (Sebio, Walchsing, F.R.G.) at a density of $1-2 \times 10^5$ cells/ml.

The cells were incubated at 37° in a humidified atmosphere of 5% CO₂-air and maintained continuously in the logarithmic growth phase by inoculating fresh medium every 2 days.

Cell staining. After 2 days of cultivation, the cell suspension was concentrated 20-fold by centrifu-

gation at 200 g, and the cells were resuspended in fresh culture medium. Samples were prepared by diluting 20 μ l of concentrated cells in 250 μ l HBS supplemented with 0.1 M glucose (Merck, Darmstadt, F.R.G.).

The samples were preincubated in a 37° water bath for 15 min before staining. Five microliters of dye solution was added. Incubation times were 15 min for both DiOC6(3) and for INDO-1, and 30 min Rho 123. Three samples were prepared for each INDO-1 experiment. Subsequently, the cells were centrifuged and resuspended in 250 μ l of dye-free HBS. INDO-1 sample 1 remained without further addition. Sample 2 received 5 μ l A23187Br and 5 μ l of calcium solution. Sample 3 received 5 μ l A23187Br and 5 μ l EDTA solution. Five microliters of FCCP or verapamil solution was added when necessary to the cell assays. Finally, 5 μ l of propidium iodide solution was added for staining the DNA of dead cells and 5 μ l of FITC stained monodispersed particles as an internal standard for cell fluorescence and volume calibration.

In control experiments designed to determine the specificity of the potential sensitive dyes, an additional 15-min incubation step was included before PI staining using 120 mM KCl in HBS for the dissipation of the transmembrane potential, or 20 μ M FCCP for the dissipation of the mitochondrial potential, respectively.

The influence of verapamil on dye uptake was tested by incubating the cells in a 22 μ M solution for 15 min before the staining procedure.

Flow cytometry. The electrical cell volume and two fluorescences of each cell were measured simultaneously in a Fluvo-Metricell flow cytometer equipped with a Cytomic 12 microprocessor data acquisition system and linked to an Interdata 74 computer (Perkin-Elmer). The cell suspension was hydrodynamically focused through the center of an 80 μ m long cylindrical orifice of 80 μ m diameter using HBS as a sheath fluid. The electrical current through the orifice was 0.51 mA. Fluorescence was excited by an HBO-100 high pressure mercury arc lamp between 300 and 400 nm for INDO-1 and 400-500 nm for both DiOC6(3) and Rho 123 respectively. The blue fluorescence of Ca²⁺-complexed INDO-1 was collected between 420 and 440 nm, and the green fluorescence of free INDO-1 and the red fluorescence of PI above 500 nm. The green fluorescence of DiOC6(3) and Rho 123 was collected between 500 and 530 nm, and the red PI-fluorescence between 550 and 700 nm.

The list mode data were stored on magnetic tape and analyzed by FORTRAN computer programs developed previously [18].

Experimental conditions for Figs 1 and 3. Figure 1: Cell volume versus green and red fluorescence of vital FLC cells stained with the transmembrane potential indicator dye DiOC6(3) (green) and of dead FLC cells stained with the DNA dye PI (red). The cells were incubated for 30 min at 37° in the presence of 0.35 μ M DiOC6(3), washed with HBS, and reincubated for 30 min at room temperature without DiOC6(3). The dead cells were then counterstained with 60 μ M PI for at least 5 min prior to the flow cytometric measurement.

Vital and dead cells, as well as cell debris and calibration particles, could be distinguished in the cloud graph (Fig. 1A). The graph was standardized to the maximum logarithmic channel content (log 379 particles), and contour lines were plotted at 10% of this level, i.e. the position of all measured cells, particles and cell debris is indicated in the cloud display.

The green versus red fluorescence display (Fig. 1B) was obtained by projecting the contents of the clouds onto the green versus the red fluorescence plane of the cube of Fig. 1A. The vital and dead cells were located into well separated areas. The graph was standardized to the maximum logarithmic channel content (log 278 particles), and contour lines were drawn at 10% intervals downwards.

The cell volume versus the fluorescence of DiOC6(3) transmembrane indicator dye in the ADM-sensitive FLC (Fig. 1C) was obtained by re-displaying the vital cells of Fig. 1B against cell volume. A majority of the cells displayed high DiOC6(3) content and could be distinguished from the calibration particles and cell debris. The contour lines were calculated as in Fig. 1B. The graph contains 11,720 cells. One logarithmic volume class corresponds to a volume of $15 \mu\text{m}^3$. The mean cell volume of the FLC cells was $615 \mu\text{m}^3$. The mean intracellular dye concentration was 0.854 arbitrary units.

The cell volume versus DiOC6(3) display of the ADM-resistant FLC in a parallel assay showed a majority of low stained cells (Fig. 1D). The display is as in Fig. 1C and contains 10,320 cells. The mean cell volume was $503 \mu\text{m}^3$, and the mean intracellular dye concentration was 0.392 arbitrary units.

Figure 3: Cell volume versus the ratio of blue to green fluorescence of FLC cells stained with the calcium indicator dye INDO-1. The vital cells were obtained by performing an analysis similar to that used for panels A and B. The fluorescence ratio was then calculated for each vital cell, and the sum of all fluorescence ratios is displayed versus the cell volume. For FLC, in addition to cell debris, a tight cluster of vital cells was observed (Fig. 3A). The mean fluorescence ratio was multiplied by 10 in the display as compared to the original data to obtain a better resolution along the ordinate. The mean fluorescence ratio was 18.8 histogram channels which corresponds to a cytoplasmic calcium concentration of $2 \mu\text{M}$ using the conversion table given in Ref. 19. The display and cell volume scale are as in Fig. 1B. The graph contains 4080 cells.

The cell volume versus INDO-1 fluorescence ratio plot of ADM-RFLC3 (Fig. 3B) shows the increased calcium content of these cells. The mean fluorescence ratio was 34.5, and the graph contains 6875 cells. The calcium was cytoplasmic because the fluorescence ratio could be lowered by incubation of the cells with the A23187Br ionophore and 2 mM EDTA. The cytoplasmic calcium concentration corresponded to $700 \mu\text{M}$.

RESULTS

Plasma membrane potentials in ADM-sensitive and -resistant cells. Using flow cytometry, cells were

stained simultaneously with the membrane potential probe, DiOC6(3) (green fluorescence), and the dye for staining the DNA of dead cells, propidium iodide (red fluorescence) which permitted the separation of vital from dead cells (Fig. 1A). The distinction between vital and dead cells was also visible in two dimensions (Fig. 1B) after the three-dimensional "clouds" of Fig. 1A were projected onto the front plane of the cube. DiOC6(3) uptake in sensitive and resistant vital cells was then analyzed according to the cell volume (Fig. 1C and D). When cells were exposed to DiOC6(3) and PI for 30 min at 37° , and then washed and immediately analyzed by flow cytometry, a majority of ADM-sensitive FLC showed a higher (approximately 4-fold) DiOC6(3) accumulation (Fig. 1C) than ADM-RFLC3 (Fig. 1D), although both cell lines stained heterogeneously.

Effects of high K^+ and FCCP on DiOC6(3) uptake. Since the plasma membrane potential of most eukaryotic cells is thought to be primarily a potassium diffusion potential [20], high extracellular potassium (120 mM) was used to depolarize the membrane potential in FLC and ADM-RFLC3. An 84% reduction of DiOC6(3) uptake was obtained when sensitive cells were incubated under these conditions (120 mM K^+) (Fig. 2). Since DiOC6(3) levels in resistant cells are low in normal K^+ medium, it is difficult to assess accurately the percentage differences when cells are incubated in high K^+ medium. Nevertheless, it appears that for resistant cells the absolute value of DiOC6(3) fluorescence in high K^+ medium was similar to that of sensitive cells in high K^+ medium.

To distinguish the contribution of mitochondrial from plasma membrane potentials on the uptake of DiOC6(3), we used FCCP which is known to specifically dissipate mitochondrial membrane potentials [21]. Sensitive cells showed a moderate reduction (27%) in DiOC6(3) uptake when co-incubated in the presence of $20 \mu\text{M}$ FCCP (Fig. 2). This agrees with the above result with high K^+ medium and indicates that less than 30% of DiOC6(3) uptake was due to mitochondrial membrane potential. Because of low DiOC6(3) uptake in resistant cells, the effects of FCCP are also difficult to quantitate. From what we were able to measure, there appeared to be no difference in drug uptake in resistant cells with or without FCCP. In the presence of both FCCP and high K^+ , DiOC6(3) accumulation in sensitive cells was reduced by 90% which is more than each treatment alone, whereas in resistant cells this decrease was minimal.

Effects of verapamil on DiOC6(3) and Rho 123 uptake. Several reports have indicated that the calcium channel blocker, verapamil, as well as a number of other agents block drug efflux in MDR cells [22, 23]. Previously, we reported that Rho 123 uptake in ADM-RFLC3 is increased substantially when cells are co-treated with $10 \mu\text{M}$ verapamil [17]. A possible explanation of this result is that Rho 123 is carried out of these resistant cells by the MDR efflux "pump" and verapamil blocks the pump, thus enhancing drug accumulation [7]. In the present study, we used verapamil to compare its effects on DiOC6(3) and Rho 123 uptake in resistant and sensitive cells. In Table 1 it can be seen that verapamil

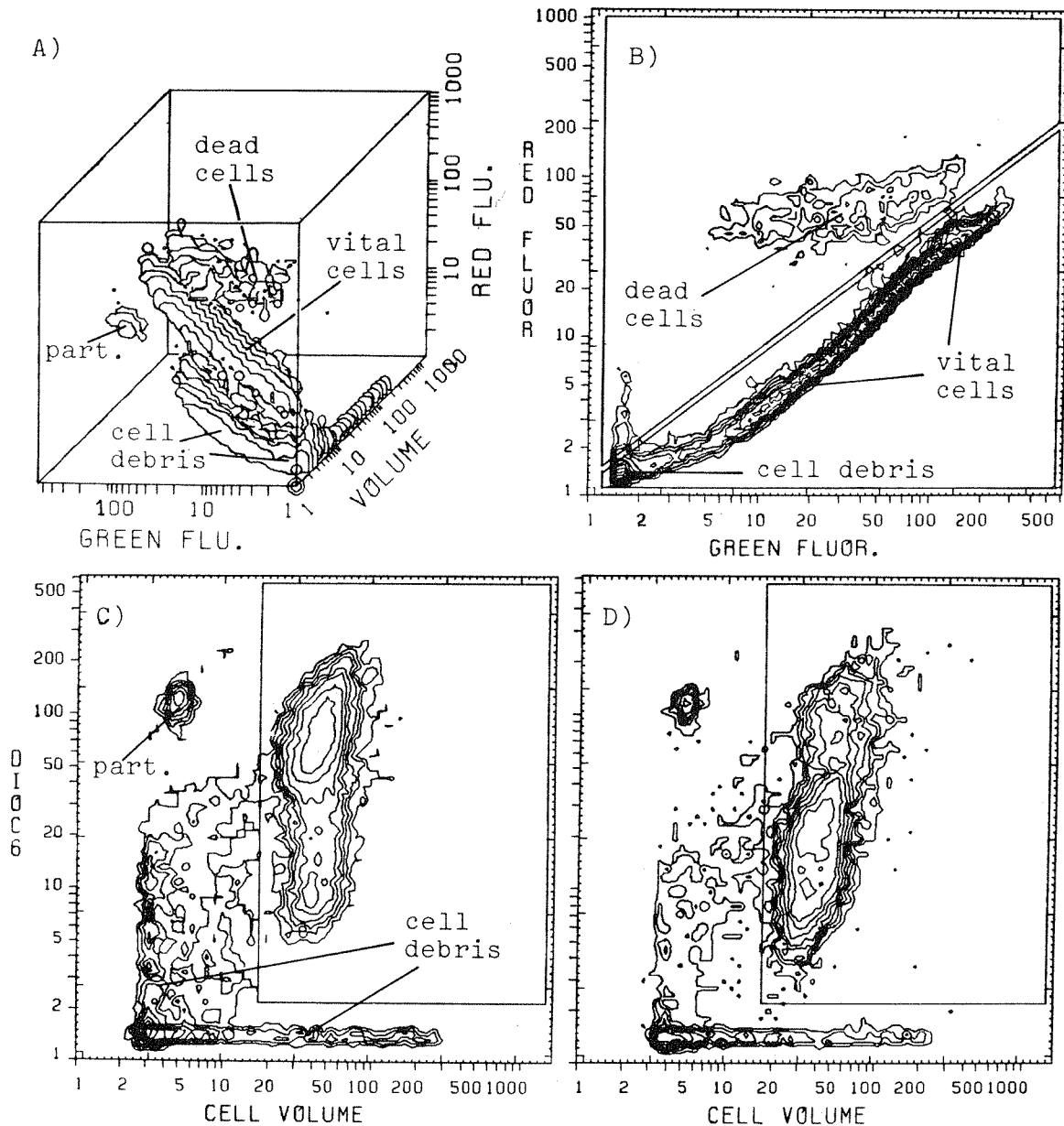


Fig. 1. Flow cytometric histograms of sensitive (A-C) and ADM-resistant (D) Friend leukemia cells (FLC) stained with the transmembrane potential indicator dye DiOC6(3) (green fluorescence) and the DNA staining dye propidium iodide (PI) (red fluorescence). Using three-dimensional analysis (A), dead cells which stain with PI can be separated from debris (based on size) and live cells which stain only with DiOC6(3). Figure 1B is derived from panel A by projecting the contents of panel A onto the green versus red fluorescence plane of the cube of panel A. By redisplaying the vital cells of panel B against cell volume it can be seen that the majority of sensitive FLC (C) accumulated DiOC6(3) to a higher degree than the ADM-resistant (ADM-RFLC3) variant (D) (for further details refer to Materials and Methods).

Table 1. Effects of verapamil on DiOC6(3) and Rho 123 uptake

	Uptake (relative fluorescent units)	
	FLC (sensitive)	ADM-RFLC3 (resistant)
DiOC6(3)	0.58	0.36
DiOC6(3) + Verapamil	1.28	0.68
Rho 123	0.27	0.04
Rho 123 + Verapamil	0.18	0.31

induced only a moderate increase in DiOC6(3) uptake in resistant cells (1.9-fold). In contrast, verapamil increased Rho 123 uptake in these cells 7.8-fold. In sensitive cells, co-treatment with verapamil slightly increased DiOC6(3) uptake and slightly lowered Rho 123 accumulation (Table 1). Thus, verapamil had a different effect on Rho 123 and DiOC6(3) uptake in both sensitive and resistant cells, which suggests a different mode of action on the uptake of these two compounds.

INDO-1/AM uptake in sensitive and resistant cells.
In addition to incubation with the positively-charged

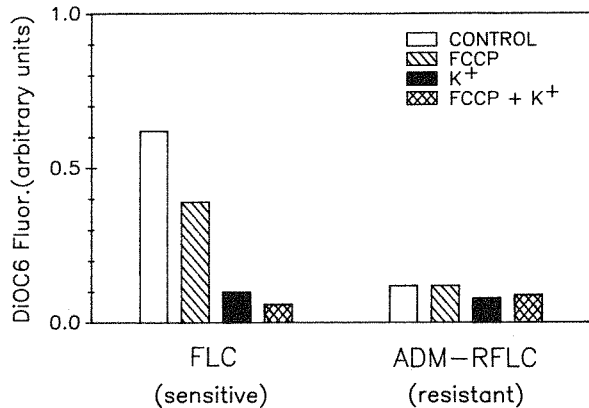


Fig. 2. Fluorescence measurement of ADM-sensitive and -resistant cells treated with the transmembrane potential indicator dye DiOC6(3) for 30 min in the presence or absence of high K⁺, FCCP or both as indicated.

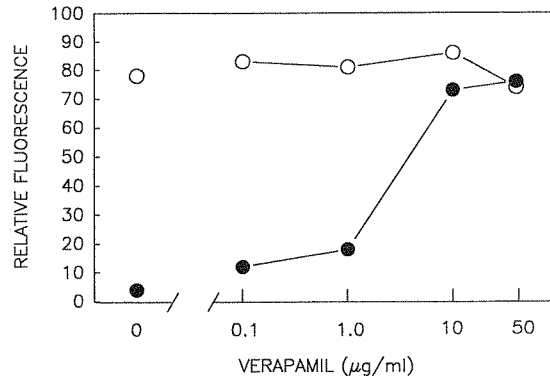


Fig. 4. Measurements of INDO-1 uptake in ADM-sensitive (○—○) and resistant (●—●) FLC when co-treated for 60 min with various concentrations of verapamil. Values represent relative fluorescence intensity at the isosbestic point of INDO-1 (432 nm). Values are the average of two separate experiments with SD < 10%.

dyes, DiOC6(3) and Rho 123 which concentrate within the cell interior due to electrically negative intracellular potentials, experiments were performed with the electrically uncharged esterase substrate INDO-1/AM. This compound crosses the cell membrane and is cleaved by cytoplasmic esterases into negatively-charged fluorescent INDO-1 molecules. As a negatively-charged molecule, free INDO-1 is trapped inside the cell.

The amount and speed of free INDO-1 accumulation in vital cells are a measure of the esterase activity of the cells. The color of emitted fluorescence light, i.e. the ratio between blue and green fluorescence, allows for the measurement of intracellular calcium concentration. Dead cells were separated by computer analysis following cell incubation with INDO-1/AM and measurement in the presence of PI. Here again, total dye uptake was higher in FLC than in ADM-RFLC3 (1.9-fold). However, the mean

blue (Ca²⁺ bound to INDO-1) to green (free INDO-1) fluorescence ratio, which indicates intracellular calcium, was markedly lower in FLC (18.8, Fig. 3A) than in ADM-RFLC3 (34.5, Fig. 3B). In the presence of 33 µM calcium ionophore A23187Br and 2 mM EDTA, the cell membrane was opened for passage of calcium ions, and all accessible intracellular calcium was chelated; the fluorescence ratio decreased slightly in FLC (17.0) but was reduced substantially in ADM-RFLC3 (23.2). These latter results are consistent with the interpretation that the high blue to green fluorescence ratio of INDO-1 in resistant cells is due to a high level of cytoplasmic calcium and that resistant ADM-RFLC3 cells contain more organelle calcium than sensitive FLC cells. When 1 mM calcium instead of 2 mM EDTA was present in the medium, together with the ionophore, the fluorescence ratio in FLC and ADM-RFLC3

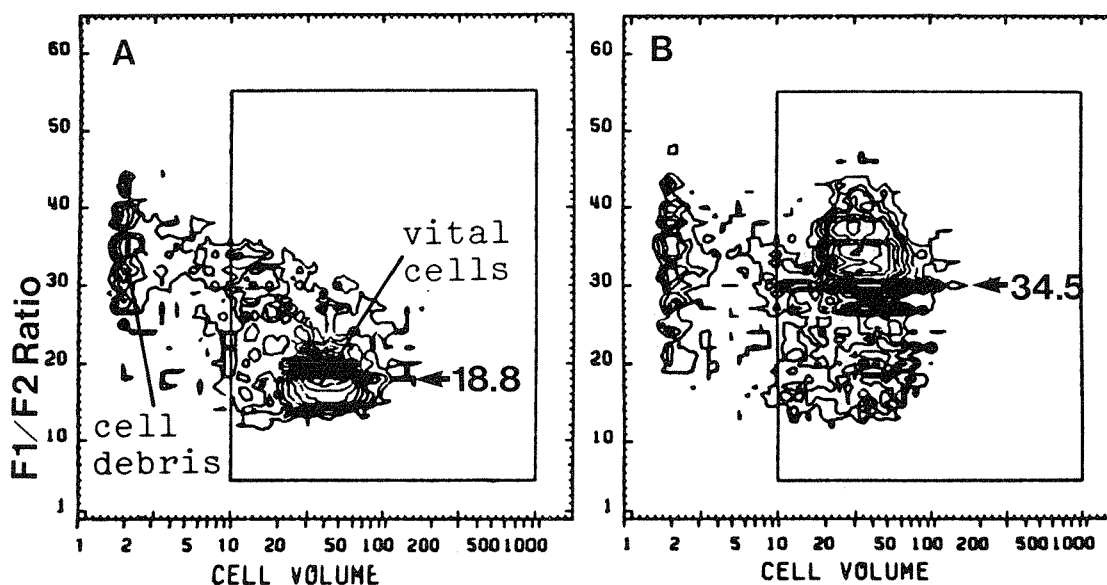


Fig. 3. Flow cytometric histograms of sensitive and resistant cells stained with calcium indicator dye INDO-1. Vital cells were obtained by performing an analysis similar to that described for panels A and B of Fig. 1. The mean fluorescence ratios were 18.8 channels for ADM-sensitive (A) and 34.5 for ADM-resistant (B) FLC.

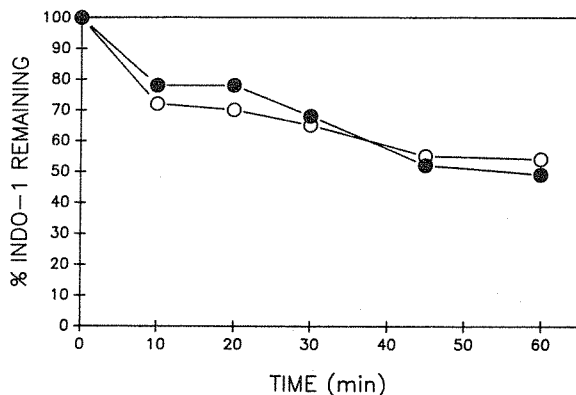


Fig. 5. Measurement of INDO-1 efflux from ADM-resistant cells in the presence (○—○) and absence (●—●) of 10 μ M verapamil.

increased to 35.5 and 37.5 respectively. This further indicates that the cytoplasmic INDO-1 dye in resistant cells was almost saturated (ratios 34.5 vs 37.5) with calcium, i.e. that resistant cells contained $> 100 \mu$ M cytoplasmic calcium.

Effects of verapamil on INDO-1/AM uptake in resistant and sensitive cells. In the presence of 10 μ M verapamil, there was a marked increase in INDO-1 accumulation (as measured at the isosbestic point) in resistant cells (13-fold as compared to INDO-1 uptake alone) with no apparent increase in sensitive cells (Fig. 4). Since in the presence of verapamil the ratio of blue to green dye fluorescence (400/470 nm) did not change (data not shown), it appears that verapamil-induced uptake did not involve the amount of free calcium available to bind INDO-1. Moreover, the efflux of INDO-1, which may simply be passive loss due to leakage, was not affected or blocked by verapamil (Fig. 5). Thus, the verapamil-induced increase of INDO-1 in these resistant cells suggests a different mechanism than that for the verapamil-induced increase of Rho 123 or other drugs identified in MDR [24].

DISCUSSION

Our finding that DiOC6(3) had an approximately 4-fold increased accumulation in ADM-sensitive as compared to -resistant FLC indicates a higher plasma membrane potential in the former cells. This is in agreement with a preliminary report in which two different sensitive tumor cell lines showed higher membrane potentials than their resistant cell counterparts [25]. To confirm that our findings in whole cells by flow cytometry were not merely due to quenching differences between these two cell types, DiOC6(3) was extracted from cells, measured by high pressure liquid chromatography, and found to be higher in sensitive than in resistant FLC (data not shown). Our experiments demonstrating that high K^+ (used to lower the plasma membrane potential) decreased DiOC6(3) uptake in sensitive cells by more than 80% further support the interpretation that DiOC6(3) accumulation is a measure of plasma membrane potentials [26, 27]. Moreover, our result with the specific mitochondrial membrane potential inhibitor FCCP, in which less than 30% of DiOC6(3)

uptake was inhibited by this agent, indicates that most of the uptake of this dye was not due to mitochondrial membrane potential. Thus, both results (high K^+ and FCCP) correlate and also agree with the interpretation that plasma membrane potentials are higher in ADM-sensitive than -resistant FLC.

The relevance of this finding to multiple drug resistance is as follows. Thus far, it is not well understood how a number of seemingly unrelated compounds could be accumulated less in MDR than in their sensitive cell counterparts [7, 9, 23]. However, we find that almost all of the compounds which show reduced accumulation in our FLC-MDR cells are positively-charged [24]. Since it is known that the electrically negative potential can act as a driving force in the attraction and retention of certain lipophilic positively-charged compounds, it is quite plausible to assume that lower plasma membrane potentials in MDR cells (than their sensitive cell counterparts) would lead to lower accumulation of positively-charged drugs. On the other hand, in the last 3 years several groups have reported on the isolation and transference of an MDR gene which confers lowered drug accumulation and drug resistance [28–32]. This gene is linked to the expression of a p170 glycoprotein that appears to be involved with the efflux (“pump”) of these positively-charged compounds [32]. How increased membrane potential could be related to this gene or glycoprotein is unknown. However, a possible relationship could exist between the appearance of this glycoprotein (“pump”) and a lowering of membrane potential. If so, then it could be expected that since K^+ permeability dictates membrane potentials in most cells and since membrane potentials of FLC responded to high K^+ , cells with MDR and/or glycoprotein p170 may express an altered permeability to potassium. Current experiments in our laboratory are focused on this point.

Previous data in a number of MDR cell types have shown that drug efflux can be blocked and sensitivity increased by co-treatment with the calcium channel blocker, verapamil [17, 22, 23]. Since DiOC6(3) did not efflux very much from sensitive or resistant FLC (data not shown) and verapamil affected its uptake much less than that of Rho 123 (Table 1), DiOC6(3) does not seem to be carried by the MDR efflux “pump.” Thus, the verapamil-induced increase in DiOC6(3) uptake seen in both sensitive (slight) and resistant (moderate) cells is more likely reflective of a true enhancement in plasma membrane potential. In contrast, the marked increase in Rho 123 accumulation in resistant cells with verapamil more likely represents a blockage of the MDR efflux “pump” which seems to be involved with the outward transport of this dye as well as a response to increased membrane potential. Since DiOC6(3) is also positively-charged, the apparent insensitivity of this compound to the efflux process of ADM-RFLC3 indicates that parameters in addition to electrical charge may be required for recognition of these compounds by the efflux transport system(s) of MDR. In fact, when we consider the differences in cellular staining between the positively-charged compounds used in this study, cells stained with DiOC6(3) appeared fluorescent all over the outer

cell membrane or entire cell (data not shown), whereas the fluorescence for Rho 123 stained cells was restricted to the mitochondria. This supports our results with high KCl and FCCP which indicate that DiOC6(3) was measuring plasma membrane potential whereas Rho 123 was more likely responsive to changes in both plasma and mitochondrial membrane potentials. The most likely explanation for the different staining capacities of these positively-charged compounds resides in their partition coefficients. Thus, it is possible that the relative lipophilicity of DiOC6(3) makes it difficult for this compound to traverse the cell membrane and it therefore remains trapped there, whereas Rho 123 easily enters the cell and is attracted by the mitochondrial membrane potential.

That the positive-charge may be an important component of MDR agrees with a recent interpretation by Beck [33] in which he suggests that a recognition component of the efflux pump is the basicity of a compound at physiologic pH. Nevertheless, it appears that, for anticancer agents as well as other compounds that are positively-charged, two separate processes may be affecting their transport and accumulation, plasma membrane potentials and active efflux. If and how these two processes are related remains to be investigated.

Although it has been shown that calcium channel blockers inhibit drug efflux in MDR cells, calcium flux itself appears to have no effect on drug efflux [34]. However, calcium levels in some MDR cells have been reported to be higher than in their parental sensitive cell counterparts [16]. Using flow cytometry, our findings of higher cytoplasmic calcium in ADM-RFLC3 ($>100 \mu\text{M}$) than in FLC ($2 \mu\text{M}$) are in accord with those that show increased Ca^{2+} in MDR cells. Even though calcium levels have not been shown to be directly responsible for the generation of cellular plasma membrane potentials, it remains possible that the differences in Ca^{2+} levels found here between ADM-RFLC3 and FLC are reflective of ion changes that could be related to electrical charge. This latter possibility, however, does not agree with preliminary data in two different resistant and sensitive cell pairs in which intracellular Ca^{2+} levels did not correlate with resistance [25]. Further studies with ADM-sensitive and -resistant cells will be required to resolve this question.

The enhanced accumulation by verapamil of the electrically negative dye INDO-1 in resistant cells, which did not involve blockage of efflux (Fig. 3), suggests a mechanism different from that described for positively-charged drugs (processed by the MDR efflux system) [4, 6]. On the other hand, since verapamil did not increase INDO-1 uptake in sensitive cells, this newly identified effect of verapamil (like its blockage of efflux) also exploits differences between resistant and sensitive cells. Since the binding of calcium to the dye was also not affected by verapamil it is possible that the enhancement of INDO-1 uptake by verapamil in resistant cells is a result of an increase in either INDO-1/AM influx or esterase activity. In regard to this latter point, Beck in a recent commentary [33] points out that the p-glycoprotein found in MDR cells could have profound effects on the packing and structural order of lipids in the plasma

membrane which may play a key role in the passage of drugs across the plasma membrane. Thus, it is possible that verapamil could be reversing this effect by direct interaction with the glycoprotein or other components of the altered MDR cell membrane.

Regardless of the mechanism(s) responsible, a new effect of verapamil on drug accumulation in MDR cells is identified here.

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