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ρ^0 Tumor Cells: A Model for Studying Whether Mitochondria Are Targets for Rhodamine 123, Doxorubicin, and Other Drugs

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ABSTRACT. A human osteosarcoma cell line devoid of mitochondrial DNA (ρ^0) and its wild-type parental cell counterpart (wt) are presented as a model to investigate drug targeting. By virtue of the absence of mitochondrial DNA, ρ^0 cells cannot perform electron transport or oxidative phosphorylation. Since most of the drugs studied are transported by the efflux pumping systems controlled by the MDR1 and MRP1 genes, both cell lines were examined for the expression of these genes, and it was found that no MDR1 and only low amounts of MRP1 were expressed. Growth inhibition experiments indicated that doxorubicin (Dox), vinblastine, and paclitaxel were equitoxic in these cell lines. On the other hand, the IC_{50} for rhodamine 123 (Rho 123) in ρ^0 cells was 50 times higher than in wt cells. This result correlates with a lower accumulation of Rho 123 in ρ^0 cells as measured by fluorescence microscopy and flow cytometry (3 times less than in wt cells). In contrast, when stained with Dox, both cell types accumulated similar amounts. Surprisingly, in these non-P-glycoprotein expressing cells, verapamil increased both Dox and Rho 123 retention. Overall, these data suggest that: (i) functional mitochondria do not appear to be targets for the growth inhibitory activities of Dox, paclitaxel, or vinblastine; (ii) for lipophilic cations like Rho 123, however, normal functioning mitochondria and maintenance of a normal mitochondrial membrane potential ($\Delta\psi_{mt}$) appear to play a critical role in the intracellular accumulation and subsequent cytotoxicities of these compounds; and (iii) verapamil increases drug accumulation in non-P-glycoprotein expressing cell lines, most likely by direct action on $\Delta\psi_{mt}$ for Rho 123 and safranin O, and on heretofore unidentified plasma membrane transporters, as well as via interaction with low levels of MRP1, for Dox. These results should be considered when Rho 123 and verapamil are used to detect P-glycoprotein. BIOCHEM PHARMACOL **60**;12:1897–1905, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. rhodamine 123; doxorubicin; ρ^0 cells; mitochondria; non-P-glycoprotein cells

Attempts have been made to identify drug targets by isolating various components of a cell and testing whether a given compound will bind and/or affect its function. Obviously, whether a drug actually uses such an identified target in an intact cell cannot be determined merely by this approach. Thus, drugs like Rho 123¶ and Dox, which have been shown to inhibit mitochondrial function [1, 2] and to bind nuclear DNA and block DNA synthesis *in vitro* [3, 4], respectively, have also been studied in intact cells to determine whether either of these drugs localizes in the corresponding cellular compartments and affects these purported targets. Clearly, in a living cell, Rho 123 localizes in the mitochondria [5], whereas Dox is found primarily in the nucleus and with time distributes somewhat in the cytoplasm [6]. However, even with the numerous studies reported on these compounds, using a variety of approaches, it still remains unclear which targets are most important for their growth inhibitory and/or cytotoxic actions in the cell.

Another complicating feature is whether either of these drugs accumulates intracellularly to a high enough level to affect their target(s). In this regard, a family of ABC transporters that include the MDR1 and MRP1 glycoproteins have been found in a wide variety of normal and tumor cells [7] and have been shown to transport Dox and Rho 123 as well as a number of diverse compounds [8, 9].

Previously, it was suggested that although Dox localizes mainly in the nucleus, it also has effects on free radical production in mitochondria that contribute to its cytotoxic activity [10]. To explore this possibility further and to learn

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¶ *Abbreviations*: Rho 123, rhodamine 123; Rho 6G, rhodamine 6G; Rho 110, rhodamine 110; Dox, doxorubicin; MDR, multidrug resistance; P-gp, P-glycoprotein; ABC, ATP binding cassette; RT–PCR, reverse transcriptase–polymerase chain reaction; $\Delta\psi_{mt}$, mitochondrial membrane potential; and wt, wild type.

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more about whether other drugs utilize functional mitochondria as a target in intact cells, a mutant osteosarcoma cell line devoid of mtDNA (ρ^0) was selected. This cell line, as compared with its wild-type parental counterpart (wt), is unable to perform electron transport and does not produce ATP by oxidative phosphorylation. Thus, this cell model is characterized and used here to address the question of the importance of normal mitochondrial function for the cytotoxic action of a number of chemotherapeutic drugs.

MATERIALS AND METHODS

Cell Types

An osteosarcoma cell line 143B (wt) was exposed to ethidium bromide for prolonged periods, and a mutant cell line with complete loss of mtDNA (ρ^0) was selected [11]. Lack of respiration was confirmed in ρ^0 cells by permeabilizing them with digitonin and adding the appropriate complex 1–4 substrates back to these cells and monitoring oxygen utilization by a Clark electrode. In the case of wt cells, oxygen consumption was stimulated with the addition of each substrate, whereas with ρ^0 cells no oxygen consumption was observed with any of the added substrates [12]. Since the ρ^0 cells are uridine and pyruvate auxotrophs, they were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/mL of uridine, 100 mM sodium pyruvate, and 10 μg/mL of gentamycin. To maintain standard experimental conditions, the parental cell line (wt) was grown in the same medium.

Growth Inhibition, Clonogenic Assays, and Drugs

For growth inhibition assays, cells (1 mL) were seeded at 4×10^4/mL for wt (doubling time = 16 hr) and 6×10^4/mL for ρ^0 (doubling time = 19 hr) in 24-well plates, and drugs were applied 24 hr later. Drug treatments were continuous for 72 hr at 37° and 5% CO_2 at which time trypan blue exclusion cell counts were conducted by hemocytometer. Inhibitory concentrations of 50% were calculated for each drug tested.

For clonogenic assays, cells (2 mL) were seeded at 150/mL for wt cells and 300/mL for ρ^0 cells in 6-well plates, and drugs were applied 24 hr later. Drug treatments were continuous for 10–14 days at 37° and 5% CO_2 at which time separate individual colonies had grown large enough to be visible by eye. Fixing the colonies (70% ethanol), rinsing in PBS, and staining with trypan blue (0.4%) allowed for calculation of cytotoxic drug concentrations.

Rho 123, Rho 6G, Rho 110, safranin O, vinblastine, Dox, paclitaxel, verapamil, Bay K8644, nifedipine, quinidine, reserpine, genestein, and 2-deoxyglucose were obtained from the Sigma Chemical Co. *N*-Trifluoroacetyladriamycin-14-valerate (AD 32) was a gift from Dr. Mervyn Israel, University of Tennessee at Memphis.

Fluorescence Microscopy

Cells were grown on 10-mm round glass discs for 24 hr and then treated with Rho 123, safranin O, or Dox at the concentrations and times indicated in the presence or absence of verapamil or other compounds at 10 μg/mL. After rinsing in drug-free medium, with and without verapamil (and other compounds), cells were mounted onto silicone rubber chambers in dye-free medium and examined for drug retention at various time points. Fluorescence was observed using an Olympus epifluorescence microscope equipped with a photomicrographic system.

Flow Cytometry

Cells were treated with Rho 123 at 10 μg/mL for 10 min with or without verapamil (10 μg/mL) then rinsed, and incubated in Rho 123-free medium with or without verapamil for 4 hr. At this time, cells were removed immediately by a rubber policeman and analyzed by FACScan flow cytometry (Becton Dickinson).

Assay of MDR1 and MRP1 Gene Expression by RT–PCR

Total RNA was purified by acid-guanidinium isothiocyanate/phenol chloroform extraction. One microgram of RNA was used for cDNA synthesis as follows: RNA was heated, chilled on ice, and then incubated with 200 U of M-MLVH reverse transcriptase, 500 nM dNTP, 10 pM random primer, and RT buffer (Gibco-BRL) for 1 hr at 37°. For PCR, the MDR1 primer was the specific sequence GGAGTGTCCGTGGATCACAAG (residue 1909–1930) and TGTTCAGGATCATCAATTCTTGT (residue 2218–2241) provided by Dr. M. T. Kuo. These primers were selected at the regions that are 36.4 and 37.5% similar to the corresponding region of MDR3 cDNA. Thus, it should not recognize the MDR3 gene. The resulting PCR product from these primers was 232 bp. For MRP1, the forward primer was CTCTGGACTGGAATGT (nucleotides 239–259) and the reverse primer was GGCATCCCTTTAAGGCT (nucleotides 673–691). GADPH was used as an internal standard, and the amplimers used for GADPH were CCACCACCCTGTTGCTAGCC (antisense) and GTCTTGACCACCCATGGAGAAGGC (sense). These primers yield a 676 bp PCR product. The PCR was carried out using the cDNA in PCR buffer according to the Perkin–Elmer Cetus protocol. Added to this mixture were 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 30 nM of the 5′ and 3′ primers, and 1.5 U of Tag polymerase. Amplification was performed in sequential cycles at 94° for 30 sec, 55° for 1 min, and 72° for 2 min for 30 cycles using a Perkin–Elmer thermocycler. The PCR product was electrophoresed in 1x TAE (TRIS-acetic-EDTA) buffer on 2% Nusieve agarose/1% agarose gel. Then the gel was stained with ethidium bromide and photographed.

RESULTS

ρ^0 Cell Model as a Tool to Determine Whether Drugs Use Functional Mitochondria as a Cytotoxic Target

Drugs that depend on functional mitochondria for localization, accumulation, or free radical formation should be less

TABLE 1. Growth inhibition assay of drugs in ρ^0 and wild-type (wt) cells

Drugs	IC_{50} (μg/mL) wt Cells	ρ^0 Cells	Ratio
Rho 123	1	50	50
Safranin O	0.35	2	5.7
Rho 6G	0.19	0.19	1.0
Rho 110	200	200	1.0
Dox	0.01	0.01	1.0
AD 32	0.10	0.25	2.5
Vinblastine	0.00075	0.00075	1.0
Paclitaxel	0.05	0.05	1.0
2-Deoxyglucose	1000	100	0.1

potent in ρ^0 than in equivalent wt cells. Thus, it is not surprising to find that in growth inhibition studies, as shown in Table 1, ρ^0 cells were 50 and 6 times more resistant than wt cells to Rho 123 and safranin O (another mitochondrial cationic dye), respectively. In contrast, Rho 6G, which is highly lipophilic, and Rho 110, which is zwitterionic, showed no difference in growth inhibition between these two cell types. ρ^0 cells were also found to be 2- to 3-fold more resistant to an anthracycline, AD 32, which has been shown previously to localize in the cytoplasm and mitochondria [6]. Moreover, the nuclear localizing chemotherapeutic agent Dox was found to be equitoxic in both cell lines, indicating that functional mitochondria are not an important site for its cytotoxic action. Likewise, the tubulin-binding agents paclitaxel and vinblastine also were found to be equitoxic in these cell lines.

We reasoned that a cell that is compromised in oxidative phosphorylation should be more dependent on glycolysis and thus be more sensitive to inhibitors of glycolysis. Indeed, our results with 2-deoxyglucose, an inhibitor of glycolysis, showed that ρ^0 cells were markedly more sensitive to this glucose analog than wt cells (Table 1).

Decreased Retention of rho 123 in ρ^0 Versus wt Cells

Although both ρ^0 and wt cells showed bright mitochondrial staining after being treated with Rho 123 at 10 μg/mL for 10 min, after 4 hr in drug-free medium most of the ρ^0 cells lost the dye, while wt cells remained relatively bright (Fig. 1, B and E). Moreover, when cells were treated with a lower concentration of Rho 123 (0.5 μg/mL for 10 min), the difference in staining between ρ^0 and wt cells could be observed immediately (Fig. 2).

Increased Rho 123 Retention in p-gp Negative ρ^0 and wt Cells by Verapamil and Other Agents

As illustrated in the top panel of Fig. 3, MDR1 gene expression could not be detected (via RT–PCR) in either cell line. Similar experiments (Fig. 3, bottom panel) demonstrated that both cell lines had minimal levels of MRP1 gene expression. However, when cells were treated with Rho 123 for 10 min at 10 μg/mL and incubated in Rho 123-free medium in the presence of verapamil, increases in drug retention were observed in both cell types (Fig. 1, D and F). These verapamil-induced increases in Rho 123 retention, therefore, are unrelated to the effects of verapamil on MDR1 and appear to be minimally or not related to MRP1. When similar experiments were performed and analyzed by flow cytometry, mean fluorescence intensity values were as follows: ρ^0 = 96, wt = 371, ρ^0 + verapamil = 1197 and wt + verapamil = 1240 (Fig. 4). From this analysis, it is clear that ρ^0 cells accumulated approximately 4 times less Rho 123 than wt cells, which confirms our fluorescence microscopy observations and agrees with previously published data reporting that ρ^0 cells have a 3- to 4-fold lower $\Delta\psi_{mt}$ than wt cells [13, 14]. Moreover, it can be seen that the effect of verapamil was more pronounced in ρ^0 than in wt cells, and the homogeneity of drug accumulation was increased in both cell types.

Several other agents were tested for their effects on Rho 123 retention in these non-P-gp expressing cells. Table 2 summarizes the results of these experiments and illustrates that both calcium channel agonists and antagonists yielded effects similar to those of verapamil on Rho 123 retention. However, nifedipine, which is known to modulate P-gp, was the lone calcium channel blocker tested that was not active in these cells. Other known modulators of P-gp, reserpine and quinidine, were found to be active in increasing Rho 123 retention in these cell lines. Genestein, a modulator of MRP1 but not P-gp, also showed no effect on Rho 123 retention in either cell type. Thus, these results further support the idea that the modulation of Rho 123 retention by verapamil and the other agents tested in these cell lines is unrelated to P-gp or MRP1.

Increased Retention of Doxorubicin by Verapamil

As expected, when treated with Dox at 10 μg/mL for 30 min and examined immediately, both ρ^0 and wt cell types stained equally bright; at 24 hr in drug free-medium most of the nuclear staining was lost. However, if co-treated with 10 μg/mL of verapamil, both ρ^0 and wt cells retained more Dox in their nuclei (Fig. 5). Thus, verapamil increases retention of nuclear, as well as mitochondrial, localizing drugs in non-P-gp expressing cells.

Increased potency of Rho 123 by Verapamil in Growth Inhibition and Clonogenic Assays

To test the functional significance of verapamil's increased retention of Rho 123 in these cell types, growth inhibition and clonogenic studies were performed. As illustrated in Table 3, verapamil increased Rho 123 toxicity in ρ^0 cells by 3.8-fold and in wt cells by 2.7-fold when the clonogenic assay was used. In contrast, when growth inhibition studies were performed, verapamil did not increase the effect of Rho 123 significantly in wt cells but did in ρ^0 cells (3.8-fold). Moreover, the difference in IC_{50} values between

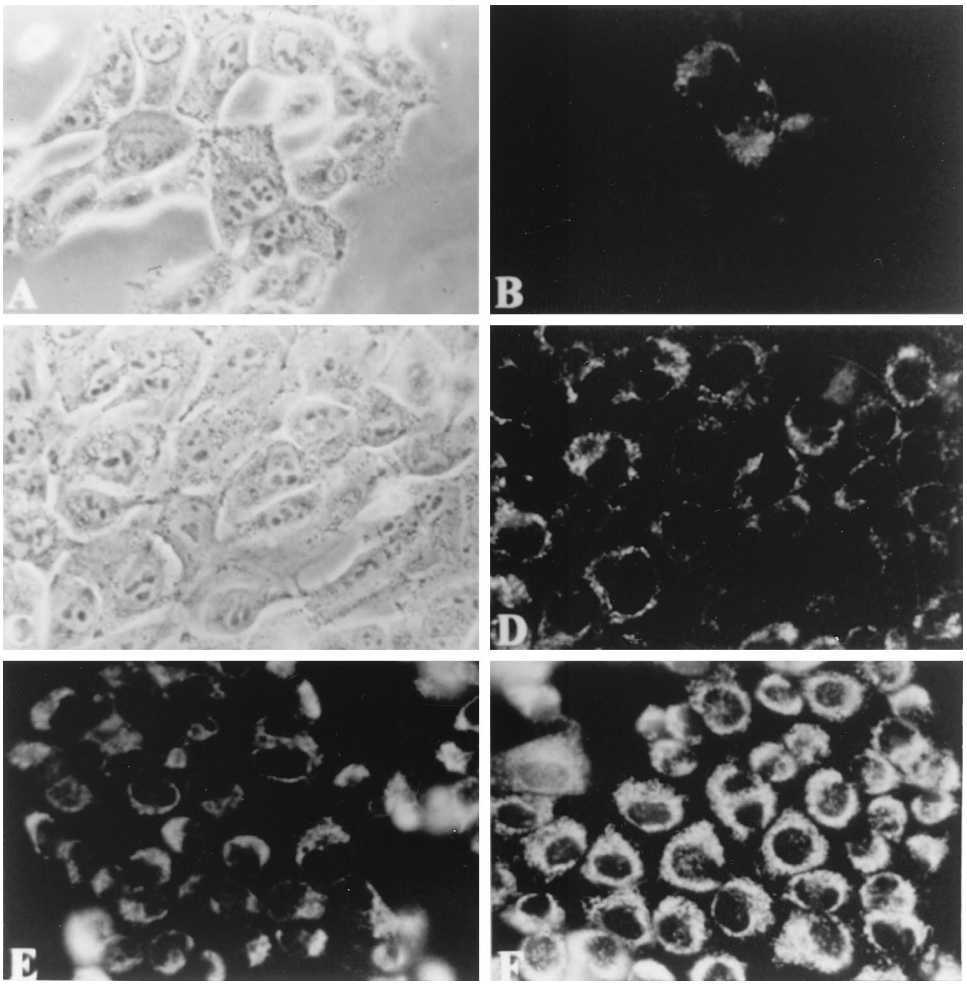

FIG. 1. Effects of verapamil on mitochondrial retention of Rho 123 in wt and ρ^0 cells. Cells were treated with Rho 123 at 10 μg/mL for 10 min with or without verapamil (10 μg/mL), and then were rinsed in Rho 123-free medium with or without verapamil for 4 hr. Phase-contrast and fluorescence micrographs of ρ^0 cells at 4 hr after Rho 123 staining (A, B) alone and (C, D) co-treated with verapamil. Fluorescence micrographs of wt cells at 4 hr after Rho 123 staining (E) alone and (F) co-treated with verapamil. Note that both ρ^0 and wt cells retained more Rho 123 when co-treated with verapamil.

ρ^0 and wt cells was more pronounced with the growth inhibitory than the clonogenic assay.

DISCUSSION

Recently, another ρ^0 and wt pair was used to study whether Dox, vinblastine, and paclitaxel utilize mtDNA as a cytotoxic target [15]. In that report, Pillay *et al.* concluded that the model they used suffers from the overexpression of P-gp in their ρ^0 cell line. Thus, transport of MDR recognizable compounds out of the cell by enhanced P-gp-mediated efflux complicates the interpretation of the cytotoxicity results they obtained, which showed that ρ^0 cells were resistant to all three drugs.

In contrast, using ρ^0 and wt cells that do not express P-gp, we found that these cells were equisensitive to vinblastine, paclitaxel, and Dox. This result suggests that none of these drugs use mtDNA or the electron transport chain as targets for cytotoxicity. To confirm the latter, we reasoned that if a drug could block electron transport and thereby inhibit oxidative phosphorylation, it will hypersensitize the cell to an inhibitor of glycolysis, 2-deoxyglucose. In fact, we had demonstrated previously that co-treating MCF-7 cells with Rho 123 and 2-deoxyglucose led to more efficient killing than either drug alone [16]. Thus, since wt cells displayed no synergistic effect when co-treated with 2-deoxyglucose and with either vinblastine or paclitaxel (data not shown), this result further supports a non-mitochondrial mechanism for each of these drugs.

Moreover, our results (Table 1) showing that ρ^0 cells were more sensitive to 2-deoxyglucose support the hypothesis that cells that are blocked in oxidative phosphorylation are more dependent on glycolysis and, therefore, are more sensitive to glycolytic inhibitors than wt cells. Indeed, dependence on glycolysis for ATP production in ρ^0 cells correlates with our findings of a relative difference of 5-fold decrease in ATP levels in this cell type as compared with wt cells (data not shown), which agrees with previous lower values reported in these cells [17].

The ρ^0 cells are devoid of mtDNA and, consequently, by

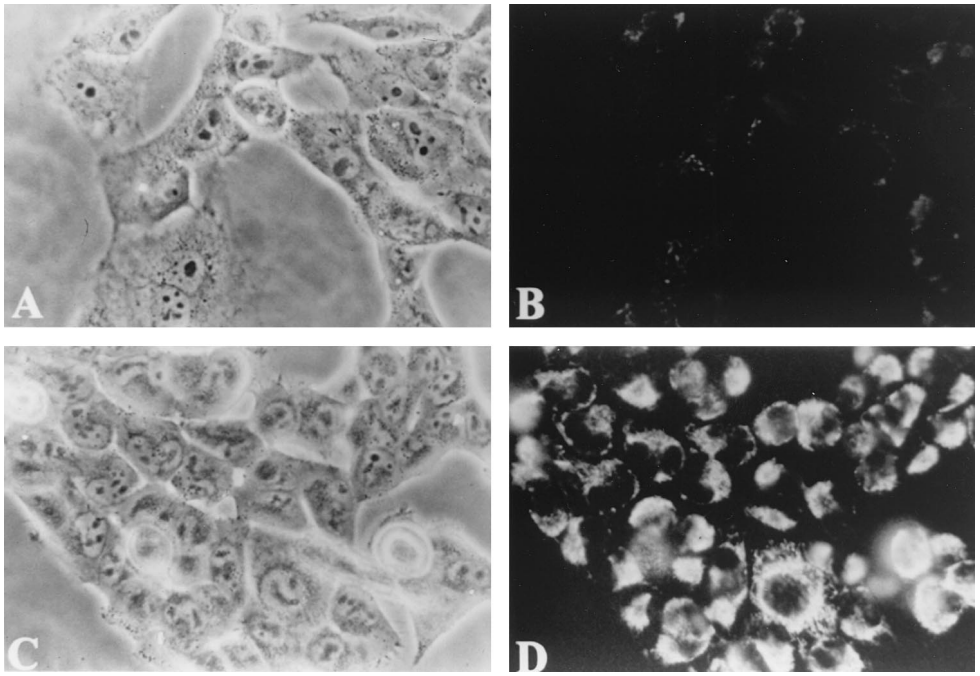

FIG. 2. Mitochondrial accumulation of Rho 123 in wt and ρ^0 cells. Cells were treated with Rho 123 at 0.5 μg/mL for 10 min, then were rinsed in Rho 123-free medium, and were observed immediately. Phase-contrast and fluorescence micrographs of ρ^0 (A, B) and wt (C, D) cells are shown. Note the significantly brighter staining in wt versus ρ^0 cells.

not forming intact respiratory chain complexes, do not undergo electron transport and oxidative phosphorylation [11, 12]. These processes are necessary for transmembrane proton pumping, which creates an electronegative membrane potential ($\Delta\psi_{mt}$) in the matrix side of the inner mitochondrial membrane. Thus, our results, which show that the majority of ρ^0 cells stained dimly while wt cells stained brightly (Fig. 2) after a 10-min exposure to Rho 123 at 0.5 μg/mL, indicate that ρ^0 cells do indeed have an $\Delta\psi_{mt,}$ albeit reduced when compared with that in wt cells.

A mechanism by which ρ^0 cells are able to generate a $\Delta\psi_{mt}$ has been proposed recently to occur via reversal of ATP transport into mitochondria through the adenine nucleotide transporter (ANT) [13]. Thus, by exchanging ADP for ATP, the internal environment of mitochondria becomes more electronegative. This process, however, does not appear to restore the $\Delta\psi_{mt}$ to its normal value [13]. Although the differences in Rho 123 staining detected between ρ^0 and wt cells most likely reflect differences in their intrinsic $\Delta\psi_{mt,}$ the speed of passive inward diffusion and the egress efficiency of the dye may also play a role.

Since ρ^0 and wt cells are P-gp negative and show similarly low levels of MRP1 in both cell types, the significant differences in Rho 123 accumulation detected in these cell lines cannot be attributed to either of these transport systems even though Rho 123 has been shown to be a substrate for P-gp [18, 19]. Moreover, the process by which ρ^0 or wt cells lose Rho 123 appears to be much slower than the P-gp-mediated efflux of this compound. Likewise, the increase in Rho 123 retention observed when either cell type was co-treated with verapamil (Figs. 1 and 4) appears to be much slower (4 hr after a 10-min incubation) and different from the effects of verapamil on P-gp.

Binding sites for calcium channel blockers have been identified on mitochondrial membranes [20, 21], and Ca^{2+} flux in and out of mitochondria has been suggested previously to be involved with changes in $\Delta\psi_{mt}$ [22]. It is possible, therefore, that verapamil could be influencing the retention of Rho 123 and another cationic dye, safranin O (data not shown), in these organelles by directly binding to mitochondrial receptor sites and thereby increasing $\Delta\psi_{mt.}$ It would therefore be reasonable to expect a greater $\Delta\psi_{mt}$ increase in ρ^0 cells than in wt cells, which would explain the flow cytometry data illustrated in Fig. 4. An alternative possibility is that verapamil is binding the newly identified ABC transporter, which has been found to be localized on the mitochondrial membrane of numerous cells and tissue types [23]. To date, there have been no reports on whether Rho 123 or other lipophilic cations are substrates for this protein, and thus this question remains to be explored.

On the other hand, the finding that retention of Dox in the nuclei of these cells is also enhanced with continuous exposure to verapamil for prolonged times indicates that verapamil is working at a site unrelated to mitochondria. In fact, Hedley *et al.* [24] reported increased daunorubicin accumulation in P-gp negative human cancer cell lines when treated with cyclosporin or ATP-depleting agents, indicating that other drug transport mechanisms distinct from P-gp were at work in these cell lines.

That verapamil differentially increases the accumulation and cytotoxicity of Rho 123 in ρ^0 versus wt cells, and that similar levels of MRP1 are measured in both, argue in favor of a direct effect of verapamil on $\Delta\psi_{mt,}$ and not via MRP1,

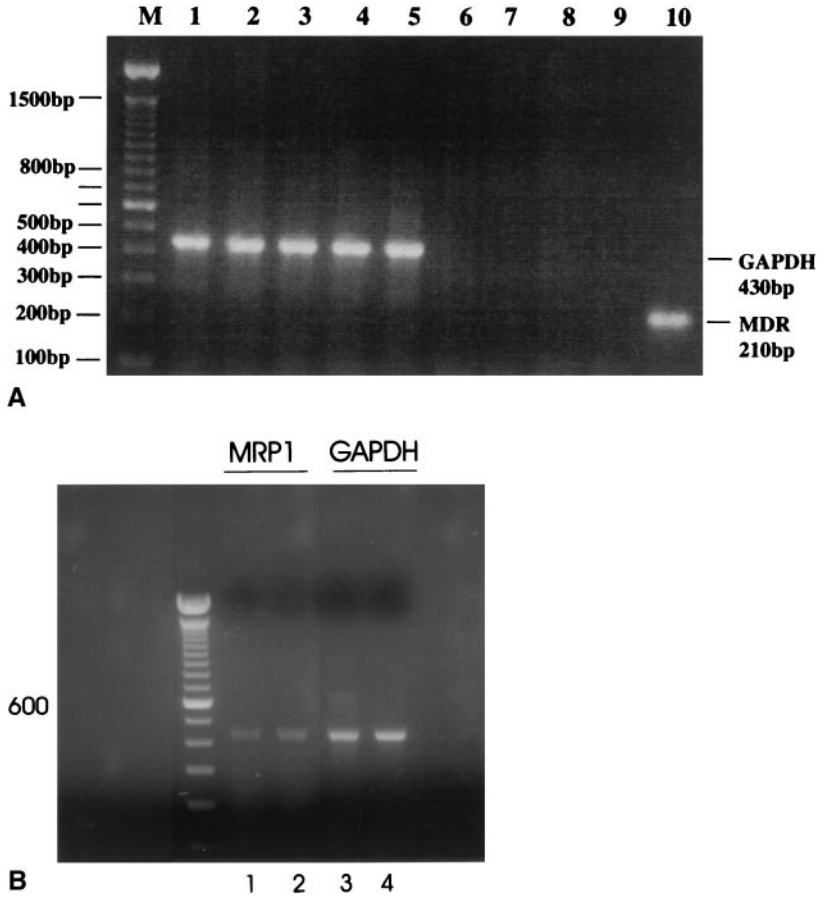

FIG. 3. (Top panel) RT–PCR analysis of MDR1 gene expression. Lanes 1–5, glyceraldehyde-3-phosphate dehydrogenase-specific (GAPDH) PCR product was used as an internal cDNA control from the following cell lines: lane 1, ρ^0 (1 μg/mL cDNA); lane 2, ρ^0 (2 μg/mL cDNA); lane 3, wt (1 μg/mL cDNA); lane 4, wt (2 μg/mL cDNA); lane 5, CEM/VBL positive control (1 μg/mL cDNA); lanes 6–10, MDR-specific PCR products from the cells in the same order as above. Note that ρ^0 and wt cells show no MDR product. (Bottom panel) RT–PCR analysis of MRP1 gene expression. Lane 1, MRP1 product of ρ^0 cells; lane 2, MRP1 product of wt cells; and lanes 3 and 4, glyceraldehyde-3-phosphate dehydrogenase-specific (GAPDH) PCR product of ρ^0 and wt cells, respectively.

to explain these results. The finding that verapamil induces a greater increase in Rho 123 accumulation and cytotoxicity in ρ^0 versus wt cells most likely reflects a greater proportional increase in $\Delta\psi_{mt}$ in ρ^0 cells. On the other hand, since Dox accumulated equally in the nuclei of both cells, the verapamil effect for this compound most likely occurs by either interacting with an unidentified transporter and/or MRP1.

Since genestein had no effect on the retention of Rho 123 in these cells (Table 2), it is unlikely that the effect of verapamil, which is known to be less effective than that of genestein on MRP1, is working via this mechanism. Moreover, the very low amounts of MRP1 expression that we found in both cell types make it less probable that verapamil and the other modulators are directly reacting with this efflux pump. However, our results cannot absolutely rule out a minor contribution of MRP1 to the results obtained when the modulators are combined with either Rho 123 or Dox. Another factor that distinguishes these effects from P-gp-mediated efflux is that nifedipine, a known blocker of P-gp [25], had no effect on Rho 123 retention in ρ^0 and wt cells.

The equal sensitivity of these two cell lines to Dox is particularly noteworthy since it has been purported that Dox, in addition to utilizing nuclear DNA as a cytotoxic target, also utilizes the mitochondrial electron transport system to form cytotoxic superoxides and free radicals [10]. Our results clearly do not support this interpretation but rather argue in favor of a non-mitochondrial-dependent mechanism for DOX toxicity. The uptake data showing similar amounts in the nuclei of both cell types correlate with equal toxicity. Moreover, with AD 32, an anthracycline that localizes in mitochondria [6], we found 2- to 3-fold resistance in ρ^0 cells, suggesting that mitochondria, at least in part, may play a role in the cytotoxic mechanism of this drug.

As shown in Table 1, ρ^0 cells were 50-fold more resistant than wt cells to Rho 123 when measured by growth inhibition assays. This difference appears to be due to the lack of the Rho 123 target, functional mitochondria, and a lowered $\Delta\psi_{mt}$ in ρ^0 cells, leading to the reduced accumulation of Rho 123. However, the difference in toxicity between these two cell lines was less when measured by clonogenic assays, which could be explained by considering

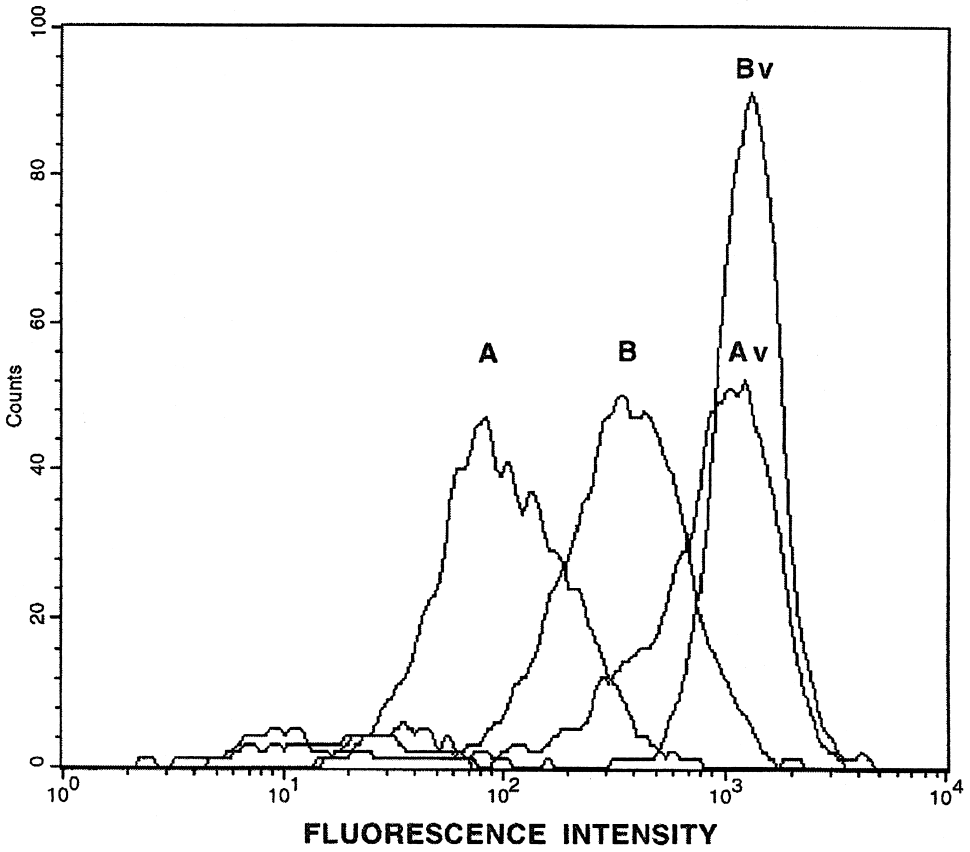

FIG. 4. Flow cytometric analysis of Rho 123 retention in ρ^0 and wt cells with or without verapamil. Cells were treated with Rho 123 at 10 μg/mL for 10 min in the absence (ρ^0 = A, wt = B) or presence (ρ^0 = Av, wt = Bv) of verapamil (10 μg/mL), then rinsed in Rho 123-free medium with or without verapamil for 4 hr, and analyzed. Note the significantly lower Rho 123 fluorescence in ρ^0 cells as compared with wt cells when treated with Rho 123 alone and increased Rho 123 staining in both cell types when co-treated and rinsed in verapamil.

that during this assay cells are exposed to Rho 123 for longer periods of time and, thus, even if ρ^0 cells have lower $\Delta\psi_{mt,}$ they eventually will accumulate enough drug to kill the cell. Since Rho 123 acts as an uncoupler in isolated mitochondria [1, 2] and is dependent upon the strength of both $\Delta\psi_{mt}$ and plasma $\Delta\psi$ for intracellular accumulation [26, 27], it is not surprising to find ρ^0 cells to be more resistant than wt cells to this drug. This, however, does not imply that Rho 123 kills the cell by inhibiting oxidative phosphorylation. It is not clear whether a normally functioning cell with intact mitochondria will die if its mitochondria become uncoupled. The fact that ρ^0 cells are able

TABLE 2. Effects of drugs on rho 123 retention in mitochondria in non-P-gp expressing cells

Drugs	Effect	MDR modulator	Known mechanism of action
Verapamil	+	+	Ca^{2+} channel antagonist (phenylalkylamine class). Closes voltage-gated Ca^{2+} channels in excitable cells. Reported to localize mitochondrial matrix in live cells. Variable data on binding directly to mitochondria and affecting Ca^{2+} flux.
Bay K8644	+	+	Ca^{2+} channel agonist (1,4-dihydropyridine class). Reported to localize in mitochondrial matrix in live cells. Variable data on binding directly to mitochondria and affecting Ca^{2+} flux. Increase in State 4 respiration in isolated mitochondria.
Nifedipine	−	+	Ca^{2+} channel antagonist (1,4-dihydropyridine class). Works best on L-type Ca^{2+} channels in smooth muscle.
Reserpine	+	+	Antihypertensive. Blocks vesicle storage of biogenic amines (norephinephrine, serotonin, dopamine) in neurons via ATP and MG^{2+} mechanism.
Quinidine	+	+	Blocks activated and non-activated Na^+ channels. Works best in depolarized tissue. Also can block K^+ channels.
Genestein	−	−	Inhibitor of tyrosine kinase. Competitive inhibitor of ATP in other protein kinases. MRP-specific modulator.

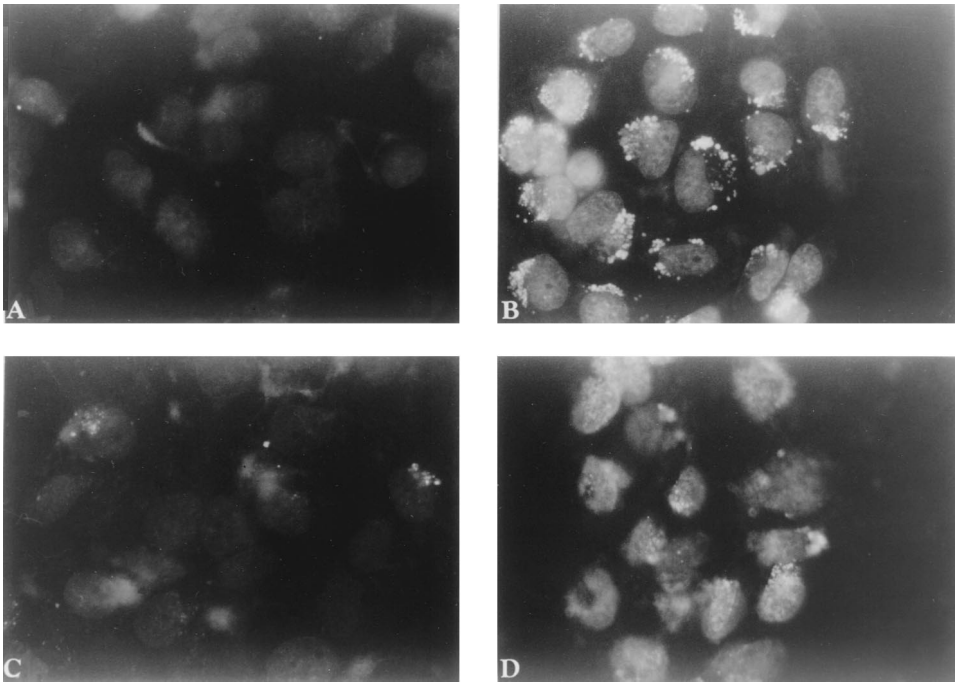

FIG. 5. Effects of verapamil on nuclear retention of Dox in wt and ρ^0 cells. Cells were treated with Dox at 10 μg/mL for 30 min with or without verapamil (10 μg/mL) and then were rinsed in Dox-free medium with or without verapamil for 24 hr. Fluorescence micrographs of wt cells at 24 hr after Dox staining (A) alone and (B) co-treated with verapamil. Fluorescence micrographs of ρ^0 cells at 24 hr after Dox staining (C) alone and (D) co-treated with verapamil. Note that both wt and ρ^0 cells retained more Dox when co-treated with verapamil.

to grow without undergoing oxidative phosphorylation argues against the concept that blockage of this process alone leads to cell death.

It can be argued, however, that ρ^0 cells have been selected to survive without oxidative phosphorylation capability and, therefore, have developed other mechanisms for this purpose. Thus, they are not equivalent to cells treated with uncouplers, which have normal mitochondria. Nevertheless, our data would be better explained by increased accumulation of the positively charged drugs, Rho 123 and safranin O, in wt versus ρ^0 cells due to the increased $\Delta\psi_{mt}$ in the former cell type. In fact, Buchet and Godinot [13] and Barrientos and Moraes [14] estimate that $\Delta\psi_{mt}$ of these ρ^0 cells is 3–4 times lower than in wt cells, which agrees with our results that show ρ^0 cells retained four times less Rho 123 than wt cells (Fig. 4). Thus, by whatever mechanism Rho 123 ultimately kills a cell, the intracellular level of drug accumulation necessary for this action is facilitated by the strength of $\Delta\psi_{mt.}$

TABLE 3. Effect of verapamil (Vpl) on Rho 123

	Clonogenic assay			Growth inhibition		
	IC_{50} (μg/mL)			IC_{50} (μg/mL)		
Cell type	Rho 123	Rho 123 + Vpl	Ratio	Rho 123	Rho 123 + Vpl	Ratio
wt	0.8	0.3	2.7	1	0.8	1.3
ρ^0	2.9	0.5	5.8	50	13	3.8

The fact that Rho 6G, a lipophilic cation that inhibits oxidative phosphorylation [28], did not show significant differences in potency in ρ^0 and wt cells could be explained by its higher degree of lipophilicity than Rho 123. This parameter could lead to drug accumulation in various cellular membrane components independent of $\Delta\psi_{mt.}$ In this regard, it has been reported previously that Rho 6G accumulates more than Rho 123 in the endoplasmic reticulum and that this accumulation difference is due to the greater lipophilicity of this compound [29]. It is conceivable then that Rho 6G could be cytotoxic to the cell by involving processes associated with the endoplasmic reticulum, such as protein synthesis.

In conclusion, our results are summarized as follows: (i) P-gp negative cell lines with (wt) and without (ρ^0) mtDNA are useful for determining whether anti-tumor or other drugs use functional mitochondria as a major cytotoxic target; (ii) the growth inhibitory or cytotoxic mechanisms of Dox, paclitaxel, or vinblastine do not appear to involve mitochondria since both wt and ρ^0 cells were equisensitive to these chemotherapeutic agents; and (iii) verapamil increased the retention of Rho 123, safranin O, and Dox in ρ^0 and wt cells without involving P-gp; in the case of Rho 123 and safranin O, this is most likely occurring via verapamil directly increasing $\Delta\psi_{mt}$ by interacting with mitochondrial receptors for calcium channel drugs, whereas for Dox, verapamil is acting at the plasma membrane on heretofore unidentified transport systems and/or on MRP1.

Thus, when Rho 123 and verapamil are used to detect P-gp, these possibilities should be considered.

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