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The Effect of Lysosomotropic Agents and Secretory Inhibitors on Anthracycline Retention and Activity in Multiple Drug-Resistant Cells

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SUMMARY

The effect of lysosomotropic agents and secretory inhibitors were compared with verapamil for their effect on the activity of doxorubicin (DOX) in multiple drug-resistant (MDR) P388 leukemia cells (P388R) and in blocking anthracycline efflux from these cells. Agents known to interact with the plasma membrane did not potentiate DOX activity in P388R cells unless these same agents were also capable of interacting with acidic compartments within the cell. The lysosomotropic detergent Triton WR-1339, for example, potentiated DOX activity in P388R cells and stimulated the net accumulation of daunorubicin (DAU) in P388R cells by inhibiting drug exodus. However, another detergent, deoxycholate, and two membrane active antibiotics, amphotericin B and filipin, had no effect on DOX activity and/or DAU efflux in P388R cells. Lysosomotropic agents such as chloroquine and secretory inhibitors such as monensin, cytochalasin B, and vinblastine all inhibited DAU efflux from P388R cells. In a MDR B16 melanoma cell line, the activity of DOX was potentiated by both verapamil and reserpine. These same two agents also inhibited melanin secretion from this same cell line. Based on these observations, we propose that secretory vesicles derived from the Golgi apparatus might be involved in the MDR phenomenon. We further suggest that drugs such as DOX might be concentrated in these acidic vesicles, where they would be released to the outside of the cell by exocytosis.

One of the major limitations in the treatment of cancer is the development of resistance to chemotherapy in patients who initially responded but have then relapsed. This problem is further exacerbated by the observation that these tumors are often cross-resistant to other drugs even though these drugs were not used in the initial treatment. Experimentally, this phenomenon of MDR, or pleiotropic drug resistance as it has been called, has been associated with a reduced drug accumulation, which is most often correlated with an enhanced drug efflux mechanism apparently common to various anticancer agents (1–4). This accelerated drug efflux can be inhibited by certain calcium channel blockers such as VER and calmodulin antagonists such as trifluoperazine (5–7), although a number of studies have now concluded that neither calcium nor calmodulin are involved in this enhanced drug exodus process and that these agents block drug efflux by some other mechanism (8, 9).

The hallmark of MDR is the overexpression of a membrane glycoprotein, termed P-gp, which is thought to be responsible for the energy-dependent nonspecific efflux of drugs from MDR cells. Recent evidence from the transfection of the gene encoding for P-gp into drug-sensitive cells and the subsequent expression of the MDR phenotype in these cells (10, 11) confirms the importance of P-gp in MDR. Thus, while it appears that P-gp is involved in MDR, its precise role in MDR remains to be determined. Two hypotheses, however, have been suggested. Several groups have proposed that P-gp functions as an energy-dependent drug efflux pump (2, 4, 12). More recently, a new hypothesis has been independently described by Beck (13) and Sehested *et al.* (14). They suggested that endosomal or lysosomal vesicles entrap drugs such as DOX or vinblastine, which are then extruded to the outside of MDR cells by fusion of these drug-containing vesicles with the plasma membrane. Neither model, however, fully accounts for the many diverse observations previously described for MDR.

In the present study, we present evidence that suggests that the secretory apparatus of the cell may be involved in MDR, and we speculate that P-gp may function in this intracellular compartment rather than at the plasma membrane to transport drugs into these vesicles where they become entrapped and extruded out of the cell by vesicle fusion with the plasma membrane.

Materials and Methods

Chemicals. [^{3}H]DAU, specific activity, 5 Ci/mmol, was purchased from New England Nuclear (Boston, MA). Amiodarone was a gift from

ABBREVIATIONS: MDR, multiple drug resistance; VER, verapamil; P-gp; P-glycoprotein; DOX, doxorubicin; DAU, daunorubicin; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TGN, trans-Golgi network.

Sanofi Research (New York, NY). Fischer's medium, RPMI 1640 medium, horse serum, and heat-inactivated fetal calf serum were purchased from GIBCO (Grand Island, NY). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell lines. P388 murine leukemia cells (P388S) and a DOX-resistant subline (P388R) obtained from Southern Research Institute (Birmingham, AL) were cultured from ascites fluids of tumor-bearing DBA/2 mice and maintained in Fischer's medium supplemented with 10% horse serum, 10 μM 2-mercaptoethanol, and 50 μg/ml gentamycin.

B16-BL-6 murine melanoma cells (B16S) (15) and a DOX-resistant B16 (B16/DOX) selected *in vitro* for resistance to DOX (16) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 25 μM HEPES and 50 μg/ml gentamycin.

Growth inhibition of B16S and B16/DOX by DOX alone or in combination with other agents was measured in a 24-well plate assay as previously described (17). Trypsinized cells were counted with a model Z_F Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida). Viability was determined by trypan blue exclusion. The concentration of drug necessary to reduce cell growth to 50% of control is expressed as the ID_{50}.

Cytotoxicity studies with P388S and P388R were carried out using a soft agar colony-forming assay. Cells were treated for 1 hr with DOX in the presence or absence of other agents as specified, washed free of drugs, and diluted appropriately in growth medium plus 0.12% agar. Colonies of control versus treated cells were counted after 8 days. The concentration of DOX necessary to reduce cell survival to 50% of the control is expressed as the IC_{50}.

Transport studies. The uptake of [^{3}H]DAU (0.25 nM [^{3}H]DAU plus 9.5 μM DAU) into P388S and P388R cells in the presence or absence of various agents was measured at 37° in Fischer's medium containing 10% horse serum and 25 mM HEPES as previously described (18). For DAU efflux, cells were preloaded with DAU for 30 min using conditions described for uptake, and drug exodus measured in the presence or absence of various compounds as previously described (18).

Results

Effect of membrane-active agents on anthracycline activity and transport in P388R. Several studies noted that agents that induce alterations in the lipid domain of cellular membranes can, like VER, potentiate drug activity in MDR cells (17–19). We therefore compared the effect of several membrane active agents with VER on DOX activity in P388R cells (Table 1). At nontoxic concentrations, the nonionic detergents Tween 80 and Triton WR-1339 were as effective as VER in potentiating DOX activity. The ionic detergent deoxycholate, at a concentration that alone resulted in 10% cytotoxicity, did not alter DOX cytotoxic activity in P388R cells. Lower and less toxic concentrations were likewise without potentiating activity (data not shown). Two polyene antibiotics, amphotericin B and filipin, which have been demonstrated to cause pore formation in cell membranes at concentrations used in the present study (20), were also without potentiating activity at slightly toxic (15% inhibition) and nontoxic doses. None of the above agents altered DOX activity in P388S cells (data not shown).

Both Triton WR-1339 and Tween 80 increased the net accumulation of DAU into P388R cells to that elevated level observed with VER (Fig. 1). In addition, the carboxylic ionophore nigericin significantly increased the net accumulation of DAU over that in untreated cells. In MDR cells, VER enhances drug accumulation by inhibiting an accelerated drug efflux (6, 8, 17). Like VER, Tween 80 and Triton WR-1339 also inhibited DAU efflux, whereas amphotericin B and deoxycholate, two agents that had no effect on drug cytotoxicity in P388R cells, did not affect drug exodus (Fig. 2).

TABLE 1

Comparison of membrane permeabilizing agents with VER on the potentiation of DOX activity in P388R cells

Cells were exposed for 1 hr at 37° to several concentrations of DOX with or without the indicated compound. Cells were washed free of drugs and plated in soft agar. The IC_{50} is defined as the concentration of DOX necessary to reduce cell survival to 50% of control. Results are the average of two experiments, each performed in triplicate, ± standard deviation. Degree of potentiation equals IC_{50} of P388R without agent divided by IC_{50} of P388R in the presence of membrane agent.

Compound	Concentration	IC_{50}	Degree of potentiation
		μM	
None		10.67 ± 1.08	
VER	5.0 μg/ml	1.20 ± 0.08	8.9
Tween 80	0.010%	1.69 ± 0.13	6.3
	0.025%	1.30 ± 0.06	8.2
Triton WR-1339	0.010%	1.41 ± 0.12	7.6
	0.025%	1.14 ± 0.09	8.4
Deoxycholate	0.004%	10.85 ± 0.82	1.0
Amphotericin B	0.5 μg/ml	10.13 ± 1.21	1.1
	1.0 μg/ml	10.95 ± 0.95	1.0
Filipin	1.25 μg/ml	9.70 ± 1.13	1.1
	2.50 μg/ml	10.72 ± 0.67	1.0

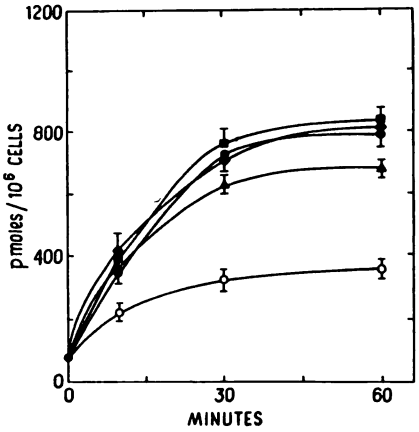

Fig. 1. Net accumulation of [^{3}H]DAU into P388R cells. Comparison of the effect of 5 μg/ml VER (●) with that of 0.025% Triton WR-1339 (♦), 0.025% Tween 80 (■), and 2 μg/ml nigericin (▲) on drug uptake. ○, Untreated P388R. Cells (2.5 × 10^6 cells/ml) were incubated with 9.5 μM DAU (containing 0.25 nM [^{3}H]DAU) in Fischer's medium supplemented with 10% horse serum and 25 mM HEPES. Incubations were terminated by rapid chilling and centrifugation. Cells were washed twice in ice-cold phosphate-buffered saline and cell pellets were dissolved in 1 N NaOH, neutralized with 1 N HCl, and counted in a Beckman liquid scintillation counter. Results are the mean of two experiments, each performed in triplicate, ± standard deviation.

Effect of lysosomotropic agents and secretory inhibitors on DAU efflux. Because nigericin stimulated DAU accumulation into P388R cells (Fig. 1), we compared the effect of nigericin and another carboxylic ionophore, monensin, as well as primary amines with VER on the potentiation of DOX activity in P388R cells (Table 2). Both nigericin and monensin potentiated the cytotoxicity of DOX in P388R cells with monensin being as effective as VER. Neither ammonium chloride nor methylamine, at concentrations reported to alter intralysosomal pH (21, 22), increased DOX activity in these cells. Both nigericin and monensin also inhibited DAU efflux from P388R cells (Table 3). In addition, although not as effective as nigericin or monensin, the proton ionophore CCCP inhibited drug efflux from resistant P388 cells (Table 3). In addition to

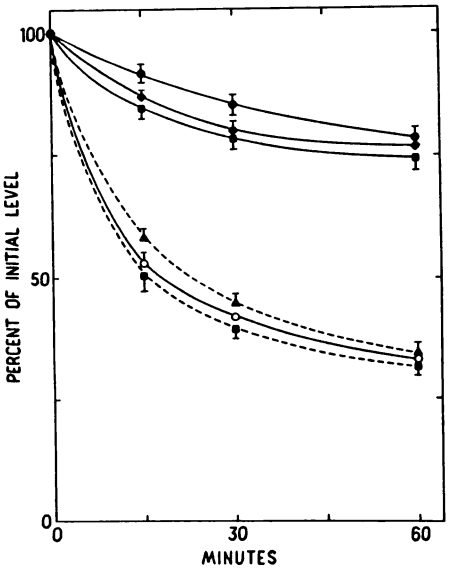

Fig. 2. Efflux of [^{3}H]DAU from P388R cells. Comparison of the effect of 5 μg/ml VER (●) with that of 0.025% Triton WR-1339 (♦), 0.025% Tween 80 (■——■), 0.004% deoxycholate (▲), and 1.0 μg/ml amphotericin B (■- - -■) on drug exodus. ○, Untreated P388R. Cells were preloaded with DAU under conditions described in legend to Fig. 1 and washed twice in ice-cold medium and DAU efflux was determined in drug-free medium as described in the legend to Fig. 1.

TABLE 2

Comparison of protein ionophores and primary amines with VER on the potentiation of DOX activity in P388R cells

Cells were exposed for 1 hr at 37° to several concentrations of DOX with or without the indicated compound. Cells were washed free of drugs and plated in soft agar. Results are the average of two experiments, each performed in triplicate, ± standard deviation.

Compound	Concentration	IC_{50}	Degree of potentiation
		μM	
None		10.0 ± 1.3	
VER	5 μg/ml	1.14 ± 0.07	8.8
Monensin	5 μg/ml	0.91 ± 0.09	11.0
Nigericin	5 μg/ml	2.19 ± 0.18	4.6
Ammonium chloride	10 mM	11.32 ± 2.1	0.9
Methylamine	10 mM	10.29 ± 1.6	1.0

TABLE 3

Comparison of the effect of proton ionophores, lysosomotropic amines, and cytoskeletal agents with VER on [^{3}H]DAU efflux from P388R cells

Cells were preloaded with DAU under conditions described in Fig. 1 and washed twice in ice-cold medium and DAU efflux was determined in drug-free medium as described in Fig. 1. Results are the mean of two experiments, each performed in triplicate, ± standard deviation.

Compound	Concentration	% of initial level after 60-min DAU efflux
None		33.0 ± 3.1
VER	5 μg/ml	78.0 ± 2.1
Quinacrine	5 μg/ml	75.0 ± 3.3
Nigericin	5 μg/ml	72.5 ± 3.2
Monensin	5 μg/ml	66.0 ± 2.3
Cytochalasin B	5 μg/ml	62.5 ± 2.4
Vinblastine	10 μM	60.8 ± 2.5
CCCP	200 μM	52.5 ± 2.9
Monodansylcadaverine	100 μM	52.0 ± 2.5
Chloroquine	100 μM	49.2 ± 2.8
Methylamine	10 mM	33.5 ± 3.0
Ammonium chloride	10 mM	32.6 ± 3.1

its effect on the pH of acidic compartments such as the lysosome or endosome, monensin has also been shown to inhibit the intracellular transport of secretory proteins (23, 24). We therefore examined the effect of other agents, demonstrated to either raise endosomal/lysosomal pH or inhibit Golgi vesicle transport of secretory proteins, on DAU efflux in P388R cells (Table 3). Quinacrine was as effective as VER in blocking DAU efflux. Monodansylcadaverine and chloroquine also inhibited drug efflux although to a lesser extent than quinacrine. Interestingly, both vinblastine and cytochalasin B partially blocked DAU efflux. However, neither methylamine nor ammonium chloride affected the enhanced drug efflux from these cells. Reducing the concentration of DAU used in the incubation medium to preload cells for efflux studies from 9.5 to 0.48 μM did not alter the inhibitory activity of lysosomotropic amines, proton ionophores, cytochalasin B, or vinblastine on DAU efflux from P388R cells (data not shown). In contrast to these results, none of the above agents altered DAU efflux from P3888 cells (data not shown).

Comparison of DOX potentiation by VER and reserpine with their inhibition of melanin secretion in B16/DOX. Unlike B16S, B16/DOX cells secrete melanin into the tissue culture medium over a 96-hr incubation period (Table 4). Both VER and reserpine inhibited this melanin secretion by greater than 10-fold. These same two agents also completely reversed B16/DOX resistance to DOX to that level observed in B16S cells (Table 4).

Discussion

The focus of numerous studies on MDR has been directed toward an understanding of how the permutations described for drug-resistant cells contribute to this phenomenon. It has become clearer from several recent studies (25, 26) that there can be multiple defects in MDR cells, the sum of which contributes to the overall level and pattern of drug resistance. In the P338R cell line, we have found that, along with the enhanced drug exodus, these cells also possess an impaired drug uptake (18, 27) and a reduced sensitivity of the cells' DNA to DNA-damaging agents (28). However, while these and other defects may participate in MDR, the hallmark of this phenomenon in cultured cells is the presence of P-gp, which is believed to be responsible for the enhanced, energy-dependent, nonspecific drug export from MDR cells.

Several groups have proposed that the decreased ability of MDR cells to retain certain drugs is due to an active efflux pump (2, 4) and, based on its primary sequence, its apparent transmembrane location, its potential nucleotide binding sites, and its homology with bacterial transport proteins, Chen *et al.* (12) also concluded that P-gp functioned as an energy-dependent drug efflux pump. Our data, however, lend additional support to a new hypothesis independently described by Sehested *et al.* (14) and by Beck (13) who suggest that the lysosomal system in the MDR cell is involved in drug exodus and that drugs such as DOX or vinblastine, which are weak bases, can become entrapped in these acidic compartments by protonation. According to this proposal, these lysosomal vesicles then migrate to the plasma membrane where they fuse and extrude their contents to the outside.

In the present study, we observed that, with the exception of ammonium chloride and methylamine, agents that are known to interact with acidic compartments such as the lysosome

TABLE 4

DOX cytotoxicity and melanin secretion in B16S and B16/DOX: effect of VER and reserpine

For IC_{50} experiments, cells were grown as previously described (18) in the presence or absence DOX with or without VER or reserpine. Results are the average of three experiments, each performed in duplicate, ± standard deviation. Melanin secretion was measured in the media from 5-day-old cultures initially seeded at 5×10^4 cells/ml (10 ml/culture). Melanin secreted into the culture media was measured spectrophotometrically at the λ_{max} (340 nM) using as a control a media blank, the pH of which was adjusted to the same pH as media from 5-day-old cultures of B16S or B16/DOX. Results are the average of three experiments, each performed in duplicate, ± standard deviation.

Cell line	DOX ID_{50}			Melanin secretion		
	Control	+VER[a]	+RES[b]	Control	+VER[a]	+RES[b]
		nM			μg/10^6 cells	
B16S	28.3 ± 2.7	14.9 ± 0.5	13.7 ± 1.1	0	0	0
B16/DOX	463 ± 31.9	29.5 ± 1.2	14.4 ± 3.5	7.56 ± 0.9	0.77 ± 0.07	0.42 ± .08

[a] 5 μg/ml VER.
[b] 5 μg/ml reserpine.

potentiated DOX activity (Table 2) and/or inhibited DOX efflux (Table 3) in P388R cells. The proton ionophores monensin, nigericin, and CCCP and the lysosomotropic amines such as chloroquine and quinacrine have previously been demonstrated to alkalinize the lysosomal compartment in several cell lines (21, 22), and all of these agents inhibited DOX efflux from P388R cells (Table 3). These findings are in accord with those recent observations by Zamora and Beck (29) and by Shiraishi *et al.* (30) who demonstrated that lysosomotropic amines such as chloroquine and other agents known to interact with the lysosome could reverse drug resistance to anthracyclines and *Vinca* alkaloids in MDR cells. Although the data from these studies as well as our own are suggestive of a role for the lysosomal system in MDR, it is also well known that many of these agents can have multiple effects on cellular functions including alterations in plasma membrane functions. Several previous studies have also demonstrated that agents that interact with the plasma membrane can also act as potentiators of drug activity in MDR cells (17, 19, 31), and we recently reported that at least for the detergent Tween 80 this potentiation was due to blocking of the enhanced drug efflux in P388R cells (18). In the present study, Tween 80 and the lysosomotropic detergent Triton WR-1339 at completely nontoxic concentrations were as effective as VER in potentiating anthracycline activity (Table 1) and inhibiting drug efflux (Fig. 2) in P388R cells. However, at slightly toxic as well as nontoxic concentrations, neither amphotericin B, filipin, nor the ionic detergent deoxycholate altered DAU uptake and/or efflux (Figs. 1 and 2) or significantly potentiated DOX activity in P388R cells (Table 1), yet both of the polyene antibiotics have been demonstrated to cause pore formation in cell membranes at concentrations used in the present study (20). Krishan *et al.* (32) also found for their P388R cells that the combination of amphotericin B plus DOX caused only a marginal additive effect on DOX cytotoxicity. It appears, therefore, that the ability of compounds to interact with plasma membrane lipids may not alone be a sufficient criterion for inhibiting drug exodus from MDR cells. Perhaps another prerequisite for inhibiting drug efflux involves the lysosome. The nonionic detergent Triton WR-1339 has been found to preferentially accumulate in the lysosomes of many cells (33), and it is possible that the other nonionic detergent, Tween 80, may also be sequestered there as well. If the hypothesis of Beck (13) and Sehested *et al.* (14) is correct, then the primary effect of these detergents may not be at the plasma membrane of drug-resistant cells, but rather in the lysosome where their concentration results in alterations in lysosomal membrane phospholipids and thereby modifies lysosomal function.

Our data, however, also suggest that rather than the lysosome system, the TGN vesicles may instead be involved in the nonspecific drug efflux phenomenon observed in most MDR cells. The TGN vesicles appear to be involved in secretion and transport of plasma membrane proteins and are reported to have an acidic pH (for review, see Ref. 34) and, hence, could entrap weak bases by protonation. This notion of a TGN involvement in MDR is supported by the following observations. First, both VER and reserpine, which potentiated DOX activity in P388R (28) and B16/DOX (Table 4) by inhibiting DOX efflux (28), also inhibited the secretion of melanin from B16/DOX (Table 4). Although it may be coincidental that the same agents that potentiate drug activity in MDR cells also block secretion, it is a striking observation that reinforces our suggestion of a TGN involvement in MDR. Moreover, in accord with this hypothesis, Sehested *et al.* (14, 35) have reported that for both MDR Ehrlich ascites and P388R, they observed an increase in plasma membrane turnover in MDR versus drug-sensitive cell lines. It is also noteworthy that reserpine is one of the classical inhibitors of the catecholamine transporter in the adrenal medulla (36). P-gp may, in fact, be similar to this nonspecific monoamine transporter in the chromaffin granules and may function like this transporter to concentrate drugs into vesicles, which would serve to protect the MDR cells from drugs until their contents could be released to the outside by exocytosis. Several laboratories (37–39) have indeed demonstrated by fluorescent microscopy anthracycline localization in discrete cytoplasmic granules in MDR cells, rather than in the nucleus as observed in drug-sensitive cells. Moreover, electron microscopic examination of several MDR cells reveals a greatly expanded Golgi compartment and Golgi-derived vesicles (40–42). Cornwell *et al.* (43) also characterized vesicles that accumulate [^{3}H]vinblastine from MDR cells and drug-sensitive cells on Percoll gradients and found that the greatest difference in [^{3}H]vinblastine accumulation between vesicles of the two cell types occurred at densities corresponding to plasma membrane and Golgi vesicles rather than lysosome. That P-gp has not been detected intracellularly by immunofluorescent microscopy is presumably due to the inability of the monoclonal antibodies to recognize incomplete glycoproteins in the interior of the MDR cell, but this clearly does not negate its presumed presence on Golgi and, for that matter rough endoplasmic reticulum, membranes.

Second, in accord with Tsuruo and Iida (44), we observed that agents that disrupted the cytoskeletal system also inhibited

DAU efflux from P388R cells (Table 3). Both of these agents are known to interfere with secretion in a wide variety of secretory cells (45) and it is possible that these agents inhibit DAU efflux from P388R cells by blocking exocytosis.

Third, we observed that monensin was as effective as VER in potentiating DOX activity in P388R cells (Table 2) and inhibiting DAU efflux (Table 3). Monensin has also been demonstrated to inhibit secretion of a variety of molecules from many different cell types, and its effect appears to be due to a block in the transport of secretory vesicles to the cell surface, leading to distention of Golgi vesicles (46–48). In fact, Rustan *et al.* (48) have recently reported that, in addition to monensin, both VER and chloroquine can also inhibit very low density lipoprotein secretion in cultured rat hepatocytes.

Fourth, neither we (Table 3) nor Zamora *et al.* (49) could demonstrate any effect of methylamine or ammonium chloride on drug efflux from MDR cells. The ability of both of these compounds to alkalinize endosomes and lysosomes appears to be universal. On the other hand, there is preliminary evidence to suggest that in some cell types secretory vesicles of the Golgi apparatus may be unaffected by methylamine or ammonium chloride (50, 51). Perhaps this is due to the varying degrees of efficiency by which charged amines are transported into certain vesicles.

Finally, in accord with previous studies (18, 31), we also observed that very low, nontoxic concentrations of two nonionic detergents can block drug efflux (Fig. 2) and potentiate DOX activity in P388R cells (Table 1). At similar concentrations, these detergents have been demonstrated to also inhibit catecholamine secretion from PC-12 pheochromocytoma cells (52), presumably by stabilization of the membrane lipid bilayer (52, 53). These findings further strengthen the possibility that certain detergents may also block DOX efflux from P388R cells by inhibiting its secretion.

Based on our data and the above considerations, we propose that, instead of the endosome or lysosome, the acidic vesicles of the TGN, which have been shown to be destined for secretion, are involved in the nonspecific, energy-dependent removal of drugs from MDR cells. We speculate that drugs that enter the cells by passive diffusion can be sequestered in these Golgi vesicles either by P-gp, which would bind and actively transport certain drugs into these vesicles, or by passive diffusion. Once inside these vesicles, the drugs would be protonated and, hence, entrapped in the acidic environment of the vesicles. This process of vesicular drug accumulation would be, in part, similar to catecholamine concentration in storage granules of the adrenal medulla and might even explain why the expression of *mdr1* mRNA in normal adrenal medulla cells is so highly elevated (54). Perhaps P-gp is related to the monamine transporter of the chromaffin granules. After drug concentration, these vesicles would then migrate to the plasma membrane where they would fuse with the plasma membrane by exocytosis and extrude their contents to the outside.

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