Effects of the glucosphingolipid synthesis inhibitor, PDMP, on lysosomes in cultured cells

Anne G. Rosenwald[1] **and Richard E. Pagano**[2]

Department of Embryology, Carnegie Institution of Washington, 115 W. University Parkway, Baltimore, MD 21210

Abstract The glucosphingolipid synthesis inhibitor, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) has a wide range of effects on cell physiology and morphology. Here, we studied the effects of high concentrations of PDMP on cells in culture and found that fluorescent analogs of PDMP targeted to the lysosomes of Chinese hamster ovary (CHO) cells. Overnight incubation of the cells in the presence of drug induced enlargement ("vacuolization") of the lysosomes. PDMP was toxic at high concentrations (> 30 μM); this finding was used to select CHO cells that exhibited increased resistance to PDMP (PDMP^R cells). The PDMP^R cells were ~2-fold more resistant to PDMP than the parental cells (CHO-P). PDMP^R cells were resistant to a number of other drugs that are also lipophilic and possess a titratable amino group. The multidrug resistance exhibited by the PDMP^R cells was distinct from that observed in cells (MDR cells) that overproduce the plasma membrane drug pump, P-glycoprotein. In addition, MDR cells were extremely sensitive to PDMP.—**Rosenwald, A. G., and R. E. Pagano.** Effects of the glucosphingolipid synthesis inhibitor, PDMP, on lysosomes in cultured cells. *J. Lipid Res.* 1994. **35:** 1232–1240.

Supplementary key words fluorescence • glucosylceramide synthase • lipophilic amine • microscopy • multidrug resistance • 1-phenyl-2-decanoylamino-3-morpholino-1-propanol

The glucosphingolipid synthesis inhibitor, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP; 1, 2), has a number of effects on cells in culture (for a review, see ref. 3). PDMP inhibits glucosylceramide synthase in several systems in vivo and in vitro, and inhibits transport of sphingolipids and proteins through the secretory pathway (4). In addition, PDMP inhibits protein synthesis (4) and DNA synthesis (5), causes alterations in cell growth (5), and modulates cell-signalling pathways (5–7). Here we show that, at high concentrations, this drug also acts as a toxic lipophilic amine on cells in culture.

Weak bases have been called lysosomotropic because of their affinity for the acidic compartments of cells, especially the lysosomes (8). At neutral pH, these compounds are uncharged and can easily cross biological membranes. However, at acidic pH, these compounds become protonated and trapped within lysosomes. Lipophilic amines (also called lysosomotropic detergents or cationic amphiphilic drugs) are also lysosomotropic. Effects of lipophilic amines include induction of lamellar body formation following inhibition of phospholipid catabolism (9) and disruption of lysosomal membranes with subsequent release of lysosomal contents into the cytosol (10–12). We found that PDMP targeted to lysosomes in Chinese hamster ovary (CHO) cells and altered lysosomal morphology. Incubation of cells in increasing amounts of PDMP was increasingly toxic, and this observation was used to select cells that were ~2-fold more resistant to PDMP than parental cells. The PDMP-resistant (PDMP^R) cells were also resistant to several other lipophilic amines. However, this type of multidrug resistance was distinct from that observed in cells that overproduce the multidrug resistance protein, gp170 (13).

MATERIALS AND METHODS

Materials

The synthesis of 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) was performed as described (1), and the D,L-*threo* and D,L-*erythro* stereoisomers were separated from each other by crystallization (2). D,L-*threo*-PDMP is a more effective inhibitor of the target enzyme, glucosylceramide synthase, than D,L-*erythro*-PDMP (2). Fluorescent analogs of D,L-*threo*-PDMP were synthesized as described (4), replacing the decanoic acid group with either 5-(5,7-dimethylpyrromethene-

Abbreviations: BSA, bovine serum albumin; C_5-DMB, 5-(5,7-dimethylpyrrometheneboron difluoride)-1-pentanoic acid; C_5-DMB-PMP, C_5-DMB-phenylmorpholinopropanol; C_{12}-NBD, 12-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoic acid; C_{12}-NBD-PMP, C_{12}-NBD-phenylmorpholinopropanol; CHO, Chinese hamster ovary; CHO-P, parental CHO cells; FBS, fetal bovine serum; HMEM, 10 mM HEPES-buffered MEM, pH 7.4, lacking phenol red; MDR, multidrug resistant; MEM, minimal essential medium; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PDMP^R, PDMP-resistant cells.

[1]Current address: Laboratory of Biological Chemistry, DCT, NCI/NIH, Bldg. 37, Room 5D-02, 9000 Rockville Pike, Bethesda, MD 20892.

[2]To whom correspondence should be addressed.

Downloaded from www.jlr.org by guest, on June 18, 2012

boron difluoride)-1-pentanoic acid (C_5-DMB) or 12-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoic acid (C_{12}-NBD), using the appropriate *N*-hydroxysuccinimidyl ester (Molecular Probes, Inc., Eugene, OR) to make C_5-DMB-phenyl-morpholino-propanol (C_5-DMB-PMP) and C_{12}-NBD-phenyl-morpholino-propanol (C_{12}-NBD-PMP), respectively. Sulforhodamine-dextran (molecular mass 70 kDa) was obtained from Molecular Probes, Inc. Stearylamine was obtained from Sigma Chemical Co. (St. Louis, MO) or from Fluka (Ronkonkoma, NY). U18666A (14) was obtained from Dr. Peter Pentchev (NIH, Bethesda, MD). All other chemicals unless otherwise noted were from Sigma. Stock solutions of lipophilic amines (PDMP, fluorescent PMPs, stearylamine, sphinganine, and U18666A) were prepared in isopropanol and diluted to the appropriate concentration in growth medium as necessary. Isopropanol concentration did not exceed 2%. Imipramine and colchicine stocks were prepared in water and diluted into growth medium.

Cells and cell culture

Normal human skin fibroblasts (5659B obtained from the Coriell Institute, Human Genetic Mutant Cell Repository, Camden, NJ) were cultured in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (15). CHO-K1 cells (Pro-background; CCL-61; American Type Culture Collection, Rockville, MD) were cultured in α-modified MEM with 5% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (4, 16). Cells resistant to U18666A ($U18^R$) and its parental cell line were obtained from Dr. Laura Liscum (Tufts University, Boston, MA) and cultured as described (17) in Ham's F-12 containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, pH 7.1, but containing 5% FBS instead of newborn calf serum. The CHO cell line, CH_5CR, which overproduces the multidrug resistance (MDR) protein, P-glycoprotein or gp170, was obtained from Dr. Victor Ling (University of Toronto, Toronto, Ontario, Canada) and grown in α-MEM containing 10% FBS, 2 mM glutamine, 10 μg/ml each adenosine and thymidine, 100 units/ml penicillin, and 100 μg/ml streptomycin (18). All cells were grown at 37°C in 95% air/5% CO_2 in a humidified atmosphere.

Cells resistant to PDMP were selected from a population of CHO-K1 cells that were cultured for more than 50 passages (CHO-P). CHO-P cells were seeded in T-150 flasks (Corning Glass Works, Corning, NY) in growth medium and allowed to attach to the substratum for 24 h. Cells were then refed with medium containing 30 μM D,L-*threo* PDMP. Cells were fed with this medium every 2–3 days for the first 10 days, then once per week thereafter. After ~6 weeks in culture, several small colonies were detected. These were pooled, grown for several passages in selective medium, then cloned. One of the fastest growing clones, 30T1, was subjected to a second round of selection in medium containing 50 μM D,L-*threo* PDMP. After several weeks, the surviving colonies were pooled, grown for several passages, then recloned. One clone, 50T1-1A, was selected for further study (here called $PDMP^R$). This cell line was grown for more than 3 months in the presence of PDMP. The drug-resistance phenotype appeared to be stable, as cells grown in the absence of drug for up to 1 month retained their ability to grow in the presence of 50 μM PDMP. All clones surviving this selection scheme had a phenotype similar to 50T1-1A (see Results). Cells that were selected with slightly different regimes or with D,L-*erythro* PDMP were also similar.

Growth assays

Growth of cells in the presence of drugs was monitored in two ways. In Growth Assay No. 1, cells were plated at a density of 1×10^5 cells per 60-mm culture dish in medium with increasing concentrations of the drug to be tested and incubated at 37°C for up to 5 days. The isopropanol concentration was constant for all samples. Each day, one dish of cells in each drug concentration was counted. Cells were removed by trypsinization and counted using a hemocytometer. In Growth Assay No. 2, cells were plated at a density of 1×10^4 cells per well of 24-well culture dishes in increasing concentrations of drug in triplicate. The isopropanol concentration was constant. After 5 days, cells were washed, then solubilized by incubation with 0.2 ml 0.5 N NaOH for 30 min at room temperature. The amount of protein per well was then determined (19) compared to a BSA standard. The protein content of wells incubated in the absence of cells, but in the presence of serum-containing medium, was determined and subtracted. The dose of drug required to obtain a 50% reduction in protein/well (ED_{50}) compared to drug-free controls was determined. The fold-resistance was then calculated by dividing the ED_{50} for $PDMP^R$ cells by the ED_{50} for parental cells. In some experiments, rather than determining protein concentration per well, monolayers were stained with methylene blue (1% in 20% ethanol).

Microscopy

For fluorescence microscopy, cells were labeled by overnight incubation with medium containing 600 μg/ml sulforhodamine-dextran as described (15, 16) or with 1 mg/ml fluorescein isothiocyanate- (FITC-)dextran as described (20). Cells were labeled with C_{12}-NBD-PMP or C_5-DMB-PMP by incubation for 30 min at 37°C in MEM containing 10 mM HEPES, pH 7.4, and lacking phenol red (HMEM) and with 2.5–5 μM fluorescent drug. Cells were labeled with daunorubicin (2 μg/ml in HMEM) by incubation for 30 min at 37°C. Labeled cells were observed using a Zeiss IM-35 microscope equipped

ASBMB JOURNAL OF LIPID RESEARCH JLR

Downloaded from www.jlr.org by guest, on June 18, 2012

with epi fluorescence optics using either a 100× planapo (1.3 n.a.) or a 63× planapo (1.4 n.a.) lens. DMB-labeled cells were viewed with appropriate optics (21). For NBD/rhodamine double-labeling experiments (as in Fig. 1), filter combinations that minimized cross-over between the NBD and rhodamine channels were used. Daunorubicin-labeled cells were viewed with rhodamine optics. Photomicrographs were obtained and processed as previously described (22). For electron microscopy, cells were processed as previously described (23).

Miscellaneous

ATP depletion of cells was performed by incubating cells in glucose-free HMEM containing 2-deoxyglucose and sodium azide as described (22). The intralysosomal pH of CHO-P and $PDMP^R$ in the absence or presence of PDMP was determined by the method of Ohkuma and Poole (20).

RESULTS

Fluorescent analogs of PDMP target to lysosomes

PDMP at low concentrations (2–10 μM) inhibits the first enzyme in the synthesis of glucosphingolipids, glucosylceramide synthase, in numerous systems (reviewed in ref. 3), including CHO cells and human skin fibroblasts in vivo (4). We previously observed that a fluorescent analog of this drug, C_5-DMB-PMP, also inhibits the target enzyme in vivo at similar concentrations (4). In addition, this analog labels the Golgi complex, the intracellular site of glucosylceramide synthase (reviewed in ref. 24), as well as phase-dark, cytoplasmic vesicles (4). To identify these latter structures, CHO cells were labeled simultaneously with both a lysosomal marker, sulforhodamine-dextran (15, 16), and a second fluorescent analog of PDMP, C_{12}-NBD-PMP (**Fig. 1**). This analog, like PDMP and C_5-DMB-PMP, inhibited glucosylceramide synthase in vivo at similar concentrations (2–10 μM). We observed that the vesicles labeled by the fluorescent dextran were also labeled with the fluorescent drug (Fig. 1, compare panels A and B). Similar results were obtained with human skin fibroblasts. We conclude that the phase-dark, cytoplasmic vesicles were lysosomes.

Overnight incubation of CHO cells with PDMP induced the formation of large phase-dark structures. If these cells were subsequently incubated with C_{12}-NBD-PMP, these structures became brightly fluorescent (Fig. 1, panel C). They appeared to be derived from lysosomes as they also contained sulforhodamine-dextran (Fig. 1, panel D). Thus, PDMP localized to lysosomes and induced a change in lysosomal morphology. Such enlarged lysosomes have been previously termed "vacuolized lysosomes" (25).

High concentrations of PDMP alter cell growth

Many lipophilic amines are cytotoxic (8, 9). Because PDMP targeted to lysosomes and is a lipophilic amine, we next determined whether PDMP was toxic to cells. Inhibition of cell growth by increasing concentrations of D, L-*threo* PDMP was observed using Growth Assay No. 1 (see Methods) (**Fig. 2,** panel A). Upon prolonged incubation with PDMP ($>$ 1 week with $\geq$ 25 μM drug), cell killing was seen as determined by two criteria. First, cell rounding and sloughing from the culture dish was observed and these floating cells were unable to reattach. Second, these floating cells did not exclude the vital dye, trypan blue. Similar results were obtained with D,L-*erythro* PDMP (data not shown), although the *erythro* isomers are not effective inhibitors of glucosylceramide synthase compared to the *threo* isomers (2), suggesting that growth inhibition was independent of glucosphingolipid synthesis inhibition. We also noted that nonconfluent cells appeared to be more sensitive than confluent cells to the effects of PDMP, as has been previously observed using other lipophilic amines (11, 25). The reason for this phenomenon is unclear.

Based on these observations, we developed a method to select cells that exhibited increased resistance to PDMP (see Methods). Several cell lines from parental CHO cells (CHO-P) that were resistant to 50 μM D,L-*threo* PDMP were obtained. One clone, 50T1-1A (here referred to as $PDMP^R$), was analyzed in detail. These cells continued to grow in 50 μM drug, although more slowly than in the absence of drug (Fig. 2, panel B). We determined by Growth Assay No. 2 (see Methods) that the effective dose for obtaining 50% inhibition of growth (ED_{50}) for D,L-*threo*-PDMP was 22 μM for CHO-P cells and for $PDMP^R$ cells was 42 μM (**Fig. 3,** panel A). Thus $PDMP^R$ cells were approximately 2-fold more resistant to D,L-*threo*-PDMP, the selective agent, than CHO-P cells.

In $PDMP^R$ cells, the fluorescent analogs of PDMP targeted to the lysosomes as was found for CHO-P cells. When $PDMP^R$ cells were grown continuously in 50 μM D,L-*threo* PDMP, vacuolized lysosomes that could be stained with C_{12}-NBD-PMP and sulforhodamine-dextran were seen (Fig. 1, panels G and H, respectively). However, growth of $PDMP^R$ cells in the absence of drug for 3 days resulted in the disappearance of these structures. Staining of the resistant cells with C_{12}-NBD-PMP labeled most intracellular membranes (Fig. 1, panel E). Note also that the lysosomes were quite small (Fig. 1, panel F). This is similar to appearance of CHO-P cells grown without drug (Fig. 1, panels A and B).

The characteristics of the vacuolized lysosomes were further investigated in the $PDMP^R$ cells grown continuously in the presence of drug. First, uptake of PDMP appeared to be a passive process, as the lysosomes could be stained with fluorescent drug at 2°C and under ATP-

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB
JOURNAL OF LIPID RESEARCH

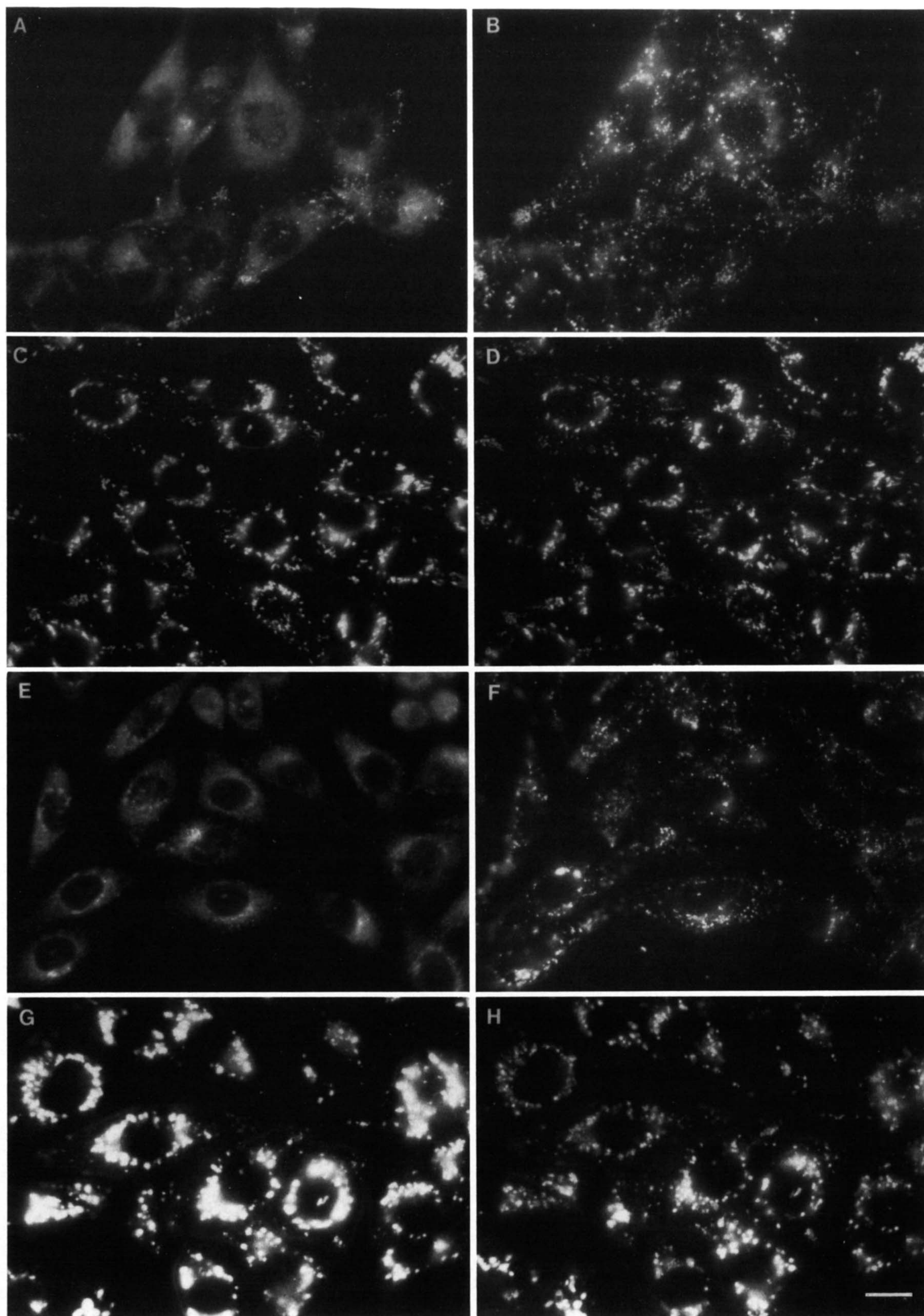

Fig. 1. CHO-P and PDMP^R cells labeled with C_{12}-NBD-PMP and sulforhodamine-dextran. CHO-P cells (panels A–D) and PDMP^R cells (panels E–H) were incubated with sulforhodamine-dextran for 18 h as described in Methods, with 0 (panels A, B, E, and F), 10 (panels C and D), and 50 μM D,L-*threo* PDMP (panels G and H; these cells had been grown continuously in the presence of PDMP). Cells were then washed and labeled for 30 min with C_{12}-NBD-PMP as described in Methods, then observed by fluorescence microscopy. On the left (panels A, C, E, and G) cells were observed in the NBD channel; on the right (panels B, D, F, and H) are the same cells observed in the rhodamine channel. Bar in lower right represents 10 μm.

Downloaded from www.jlr.org by guest, on June 18, 2012

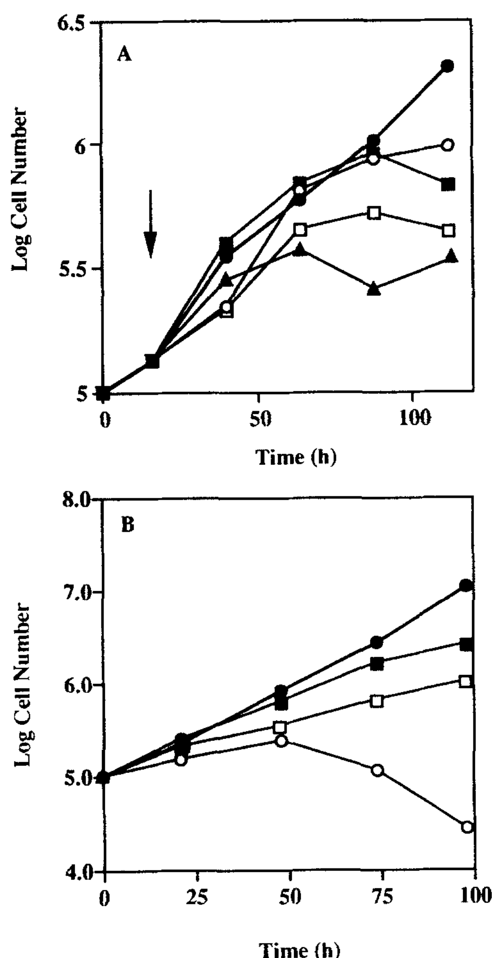

Fig. 2. Growth of CHO-P and PDMP^R Cells in D,L-*threo* PDMP. Cells were plated and grown for up to 5 days by Growth Assay No. 1 in Methods. Panel A: CHO-P cells grown in the presence of increasing amounts of D,L-*threo* PDMP, (●) 0 μM, (○) 20 μM, (■) 30 μM, (□) 40 μM, and (▲) 50 μM. In this experiment, all cells were plated initially in medium lacking PDMP. Arrow denotes refeeding of cells with the indicated concentrations of PDMP. Each point represents a single determination. Two other independent experiments gave similar results. Panel B: Comparison of CHO-P and PDMP^R cells grown in the absence or presence of 50 μM D,L-*threo* PDMP, (●, ○) CHO-P cells, (■, □) PDMP^R cells. Filled symbols are in the absence of PDMP, open symbols are in the presence of PDMP. In this experiment, cells were initially plated in the indicated concentrations of PDMP.

depletion conditions.[3] Second, by confocal microscopy, three-dimensional analysis of these structures showed that they were tubular and arrayed around the nucleus perpendicular to the substratum. Third, analysis of the lysosomes by thin-section electron microscopy revealed that the lysosomes contained large amounts of osmophilic material (**Fig. 4,** panel B). This may be a result of PDMP accumulation at this site, but may also be the result of impaired lipid hydrolysis in the presence of a lipophilic amine (9). These structures were not detected in cells grown in the absence of drug (Fig. 4, panel A).

PDMP-resistant cells are resistant to other lipophilic amines

As a number of toxic lipophilic amines induce vacuolization of lysosomes (11, 12, 25, 26), we explored the possibility that the PDMP^R cells were resistant to other lipophilic amines. One agent, U18666A, inhibits cholesterol efflux from lysosomes (14). PDMP^R cells were grown in increasing amounts of U18666A (Fig. 3, panel B). The ED_{50} for PDMP^R cells (7.5 μg/ml) was approximately 2-fold greater than that for CHO-P cells (3.3 μg/ml). PDMP^R cells were also ~2-fold more resistant to three other lipophilic amines, D,L-*erythro*-PDMP, imipramine, and sphinganine (data not shown). Another

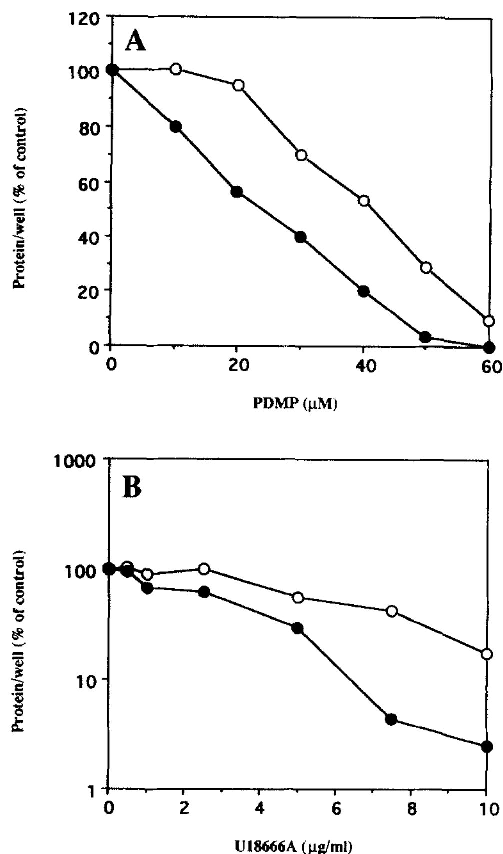

Fig. 3. Growth of CHO-P and PDMP^R cells in lipophilic amines. Cells were plated and grown in 0–60 μM D,L-*threo*-PDMP (panel A) or 0–10 μg/ml U18666A (panel B) for 5 days according to Assay No. 2 described in Methods. Results are expressed as the average percent (n = 3) of protein/well of cells grown in the absence of drug (CHO-P, ●; PDMP^R, ○).

[3]Under the ATP-depletion conditions used, the cells were very fragile and rounded. Thus, we were unable to determine the amount of drug that targeted to the lysosomes in cells treated this way compared to control cells. However, by microscopy, we observed punctate fluorescence under these conditions. We conclude that depletion of ATP did not affect the initial targeting of the drug to these structures. It is likely that a proton-gradient is required to keep such drugs trapped in lysosomes (8).

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

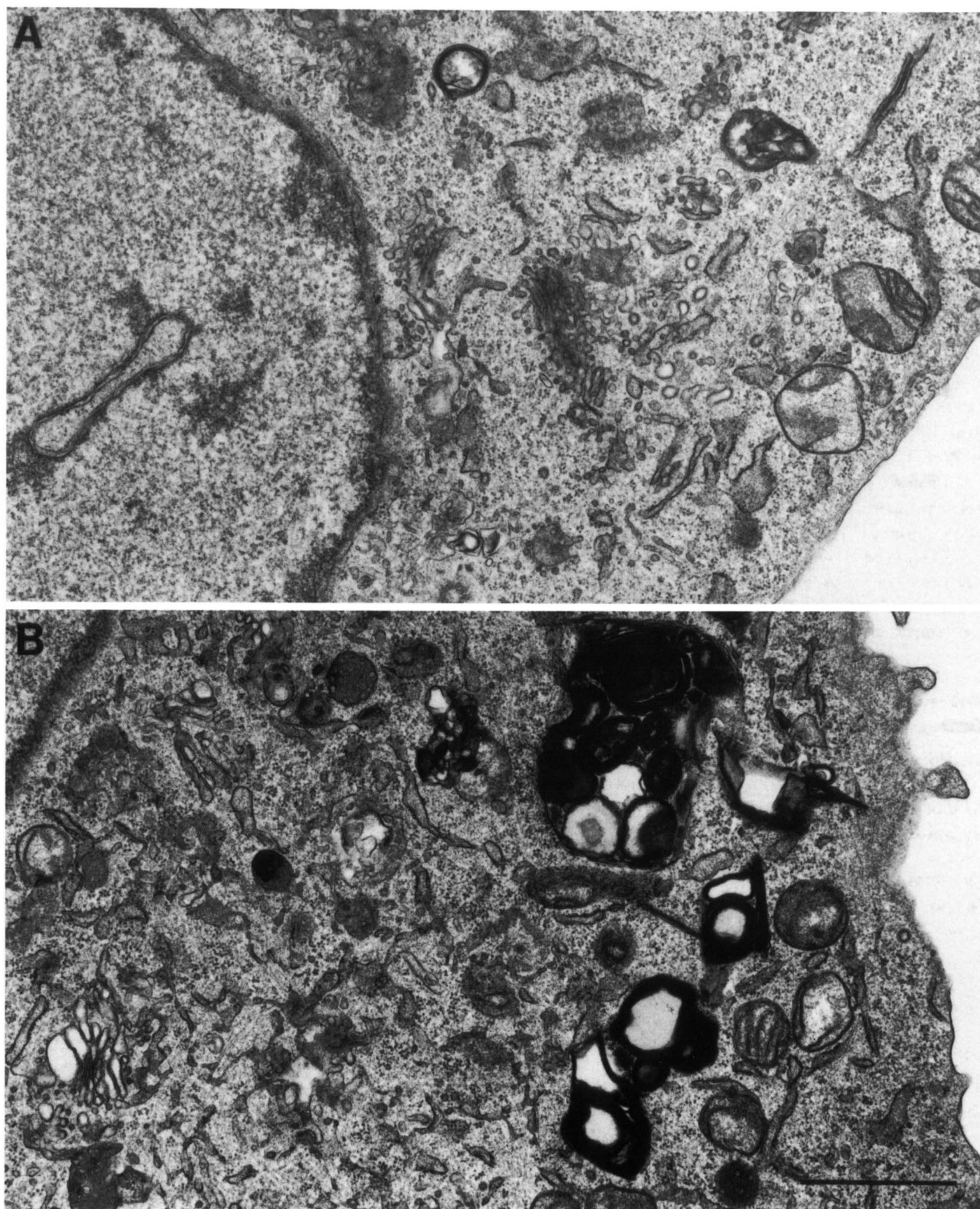

Fig. 4. Analysis of PDMP^R cells by thin-section electron microscopy. CHO-P cells (panel A) grown in regular growth medium and PDMP^R cells (panel B) grown in medium containing 50 μM PDMP were prepared for electron microscopy as described in Methods. Similar results to those shown in panel A were obtained when PDMP^R cells were grown in the absence of PDMP. Similar results to those shown in panel B were obtained when CHO-P cells were incubated overnight in medium containing 10 μM PDMP. Bar in lower right represents 1 μm.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH

lipophilic amine, stearylamine, was also tested, but PDMP[R] cells were only slightly resistant compared to CHO-P cells (1.2-fold). This suggests that stearylamine may kill cells by another mechanism than that observed with PDMP and the other lipophilic amines. We also found that cells selected for their resistance to the *erythro* isomers of PDMP exhibited resistance to the *threo* isomers and cells selected for their resistance to U18666A (17) exhibited resistance to D,L-*threo*-PDMP.

At present, the mechanism conferring resistance to the PDMP[R] cells is unclear. One possible explanation, that acidification of the lysosomal compartments is altered, as was previously shown for the chloroquine-resistant Swiss 3T3 cell line, CHL60-64 (26), does not appear to explain this phenomenon in PDMP[R] cells. Preliminary results have suggested that there is no defect in acidification in the PDMP[R] cells as measured by the uptake of FITC-dextran, a pH-sensitive marker for acidic compartments (20). Both CHO-P and PDMP[R] cells grown in the absence of PDMP were able to acidify lysosomes to approximately the same pH, while in the presence of PDMP, the intralysosomal pH rose to approximately the same level in the two cell types. Instead, there may be changes in the composition of lysosomal membranes in the PDMP[R] cells compared to CHO-P cells. Further experimentation will be required to determine this point.

PDMP-resistant cells exhibit a novel form of multidrug resistance

Multidrug resistant (MDR) cells exhibit cross-resistance to a number of different hydrophobic drugs as a result of induction of the plasma membrane drug pump, P-glycoprotein or gp170 (13). To probe the differences between this type of multidrug resistance and that observed in the PDMP[R] cells, the responses of the MDR cells (CH_5CR; 18) and PDMP[R] cells to both PDMP and colchicine (a substrate for P-glycoprotein) were compared (**Fig. 5,** panels A and B). As expected, PDMP[R] cells were resistant to PDMP and MDR cells were resistant to colchicine. However, while PDMP[R] cells were slightly sensitive to colchicine, the MDR cells were very sensitive to PDMP. Further evidence for distinct mechanisms of multidrug resistance in the two cell lines was obtained by staining with the fluorescent drug, daunorubicin, which targets to nuclei and becomes intercalated in nuclear DNA (13). No daunorubicin staining of MDR cells was detected. CHO-P cells and PDMP[R] cells, however, exhibited nuclear staining (data not shown).

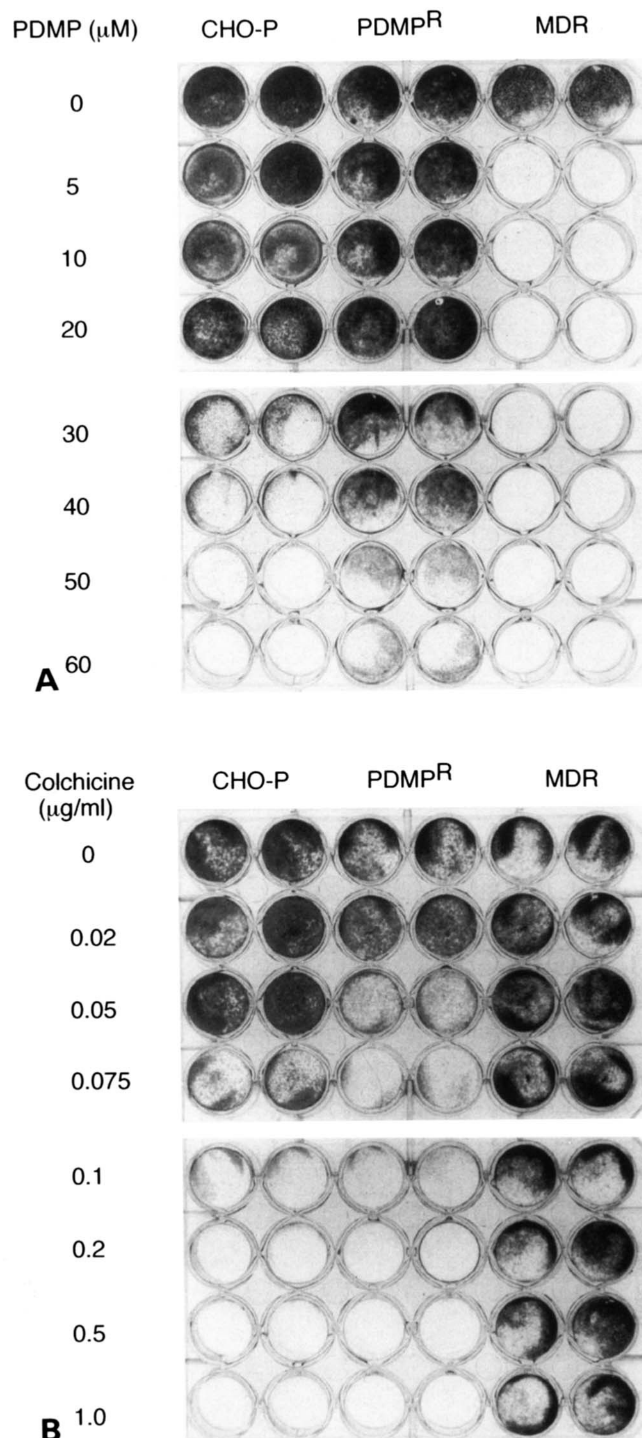

Fig. 5. Growth of CHO-P, PDMP[R], and MDR cells in PDMP and colchicine. Cells were plated and grown in 0–60 μM D,L-*threo*-PDMP (panel A) or 0–1 μg/ml colchicine (panel B) for 5 days, then fixed and stained with methylene blue (see Methods).

DISCUSSION

In this study, we have shown that PDMP targeted to lysosomes in cultured cells and induced vacuolization of these organelles. The induction of vacuolized lysosomes upon incubation of cells with amines has been previously described (11, 12, 25, 26) and is thought to result from an influx of water to the lysosomal compartment to counter-

Downloaded from www.jlr.org by guest, on June 18, 2012
ASBMB JOURNAL OF LIPID RESEARCH

act the increased osmolarity within lysosomes resulting from the trapped amine (27). Here, we demonstrate conclusively that the inducing agent was localized to the lysosomal compartment using fluorescent analogs of PDMP, C_5-DMB-PMP and C_{12}-NBD-PMP. It is interesting to note that PDMP and other lipophilic amines induce a phenotype much like that seen in Niemann-Pick, type C disease (28), a disorder in which cholesterol efflux from the lysosomes is disturbed.

The mechanism for cytotoxicity of PDMP appears to be similar to that described by Miller et al. (11), who found that high concentrations of the lipophilic amine, *N*-dodecylimidazole, were toxic. Toxicity was ascribed to the release of lysosomal enzymes, particularly proteases, into the cytosol (12). The authors proposed that the amine acts as a detergent to solubilize the lysosomal membranes and PDMP may have similar detergent-like effects. As stated previously, the fact that cells with a similar phenotype were selected with both the *threo* isomers and the *erythro* isomers of PDMP argues that the physical properties of these drugs (lipophilic amines) rather than their biological properties (enzyme inhibitors) were involved in this phenomenon.

The toxicity of PDMP permitted the selection of $PDMP^R$ cells. All of the resistant cells selected by this scheme had the same phenotype, alteration of lysosomal structure. Theoretically, one might expect to detect other forms of resistance, including overproduction of the target enzyme, glucosylceramide synthase, as has been observed using methotrexate and other drugs (29). None of the cell lines generated in the course of this study had alterations in glucosylceramide synthase activity. Moreover, preliminary results suggested that the glucosylceramide synthase activity in the $PDMP^R$ cells remained fully sensitive to PDMP. From this it appears that inhibition of glucosphingolipid synthesis in itself may not be cytotoxic to CHO cells.

Several other cell lines have been selected with lipophilic amines. These include $U18^R$ CHO cells, selected with U18666A (17), SR-8 CHO cells, selected with sphingosine (30), and CHL60-64 Swiss 3T3 cells, selected with chloroquine (26). Here, we described the selection of CHO cells with D,L-*threo*- and D,L-*erythro*-PDMP. Three of these cell lines (ones selected with D,L-*threo*-PDMP [$PDMP^R$], D,L-*erythro*-PDMP, and U18666A [$U18^R$]) demonstrated resistance to at least one other amine, suggesting that this form of resistance may be a common response to drugs of this type.

Resistance to PDMP was distinct from that observed in MDR cells, which overproduce the plasma membrane drug pump, P-glycoprotein. The extreme sensitivity of MDR cells to PDMP suggest that the combination of lipophilic amines and P-glycoprotein substrates may exhibit synergism. Since acquisition of multidrug resistance by tumors is a serious problem associated with many chemotherapeutic agents, this potential synergism seen with PDMP may be of clinical importance.

We wish to thank Drs. Victor Ling (University of Toronto, Toronto, Ontario, Canada) and Laura Liscum (Tufts University, Boston, MA) for providing CH_5CR (MDR) and U18 cells, respectively. We also thank Drs. Douglas Fambrough, Ann Hubbard, Sharon Krag, Peter Pentchev, and April Robbins for helpful suggestions about this work. Finally, we thank Ona Martin and the other members of the Pagano laboratory for their comments about the manuscript. AGR is supported by Damon Runyon-Walter Winchell Cancer Research Fund, DRG-1067. REP is supported by PHS grant R37 GM22942.

Manuscript received 21 September 1993 and in revised form 3 February 1994.

REFERENCES

1. Vunnam, R. R., and N. S. Radin. 1980. Analogs of ceramide that inhibit glucocerebroside synthetase in mouse brain. *Chem. Phys. Lipids.* **26:** 265–278.
2. Inokuchi, J-i., and N. S. Radin. 1987. Preparation of the active isomer of 1-phenyl-2-decanoylamino-3-morpholino-1-propanol, inhibitor of murine glucocerebroside synthetase. *J. Lipid Res.* **28:** 565–571.
3. Radin, N. S., J. A. Shayman, and J-i. Inokuchi. 1993. Metabolic effects of inhibiting glucosylceramide synthesis with PDMP and other substances. *Adv. Lipid Res.* **26:** 183–213.
4. Rosenwald, A. G., C. E. Machamer, and R. E. Pagano. 1992. Effects of a sphingolipid synthesis inhibitor on membrane transport through the secretory pathway. *Biochemistry.* **31:** 3581–3590.
5. Shayman, J. A., G. D. Deshmukh, S. Mahdiyoun, T. P. Thomas, D. Wu, F. S. Barcelon, and N. S. Radin. 1991. Modulation of renal epithelial cell growth by glycosylceramide: association with protein kinase C, sphingosine, and diacylglycerol. *J. Biol. Chem.* **266:** 22968–22974.
6. Felding-Habermann, B., Y. Igarashi, B. A. Fenderson, L. S. Park, N. S. Radin, J-i. Inokuchi, G. Strassmann, K. Handa, and S-i. Hakomori. 1990. A ceramide analogue inhibits T cell proliferative response through inhibition of glycosphingolipid synthesis and enhancement of *N,N*-dimethylsphingosine synthesis. *Biochemistry.* **29:** 6314–6322.
7. Shayman, J. A., S. Mahdiyoun, G. Deshmukh, F. Barcelon, J-i. Inokuchi, and N. S. Radin. 1990. Glucosphingolipid dependence of hormone-stimulated inositol trisphosphate formation. *J. Biol. Chem.* **265:** 12135–12138.
8. de Duve, C., T. de Barsy, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoof. 1974. Lysosomotropic agents. *Biochem. Pharmacol.* **23:** 2495–2531.
9. Reasor, M. J. 1989. A review of the biology and toxicologic implications of the induction of lysosomal lamellar bodies by drugs. *Toxicol. Appl. Pharmacol.* **97:** 47–56.
10. Firestone, R. A., J. M. Pisano, and R. J. Bonney. 1979. Lysosomotropic agents. 1. Synthesis and cytotoxic action of lysosomotropic detergents. *J. Med. Chem.* **22:** 1130–1133.
11. Miller, D. K., E. Griffiths, J. Lenard, and R. A. Firestone. 1983. Cell killing by lysosomotropic detergents. *J. Cell Biol.* **97:** 1841–1851.

Downloaded from www.jlr.org by guest, on June 18, 2012

12. Wilson, P. D., R. A. Firestone, and J. Lenard. 1987. The role of lysosomal enzymes in killing of mammalian cells by the lysosomotropic detergent *N*-dodecylimidazole. *J. Cell Biol.* **104:** 1223–1229.
13. Endicott, J. A., and V. Ling. 1989. The biochemistry of P-glycoprotein multidrug resistance. *Annu. Rev. Biochem.* **58:** 137–171.
14. Liscum, L., and J. R. Faust. 1989. The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one. *J. Biol. Chem.* **264:** 11796–11806.
15. Koval, M., and R. E. Pagano. 1990. Sorting of an internalized plasma membrane lipid between recycling and degradative pathways in normal and Niemann-Pick, type A fibroblasts. *J. Cell Biol.* **111:** 429–442.
16. Koval, M., and R. E. Pagano. 1989. Lipid recycling between the plasma membrane and intracellular compartments: transport and metabolism of fluorescent sphingomyelin analogues in cultured fibroblasts. *J. Cell Biol.* **108:** 2169–2181.
17. Liscum, L., and G. J. Collins. 1991. Characterization of Chinese hamster ovary cells that are resistant to 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one inhibition of low density lipoprotein-derived cholesterol metabolism. *J. Biol. Chem.* **266:** 16599–16606.
18. Ling, V., and L. H. Thompson. 1973. Reduced permeability in CHO cells as a mechanism of resistance to colchicine. *J. Cell. Physiol.* **83:** 103–116.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
20. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* **75:** 3327–3331.
21. Pagano, R. E., O. C. Martin, H. C. Kang, and R. P. Haugland. 1991. A novel fluorescent ceramide analogue for studying membrane traffic in animal cells: accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor. *J. Cell Biol.* **113:** 1267–1279.
22. Martin, O. C., and R. E. Pagano. 1987. Transbilayer movement of fluorescent analogs of phosphatidylserine and phosphatidylethanolamine at the plasma membrane of cultured cells: evidence for a protein-mediated and ATP-dependent process(es). *J. Biol. Chem.* **262:** 5890–5898.
23. Rosenwald, A. G., and R. E. Pagano. 1993. Inhibition of glycoprotein traffic through the secretory pathway by ceramide. *J. Biol. Chem.* **268:** 4577–4579.
24. Rosenwald, A. G., and R. E. Pagano. 1993. Intracellular transport of ceramide and its metabolites at the Golgi complex: insights from short-chain analogs. *Adv. Lipid Res.* **26:** 101–117.
25. Cain, C. C., and R. F. Murphy. 1986. Growth inhibition of 3T3 fibroblasts by lysosomotropic amines: correlation with effects on intravesicular pH but not vacuolation. *J. Cell. Physiol.* **129:** 65–70.
26. Cain, C. C., and R. F. Murphy. 1988. A chloroquine-resistant Swiss 3T3 cell line with a defect in late endocytic acidification. *J. Cell Biol.* **106:** 269–277.
27. Cain, C. C., D. M. Sipe, and R. F. Murphy. 1989. Regulation of endocytic pH by the Na^+, K^+-ATPase in living cells. *Proc. Natl. Acad. Sci. USA.* **86:** 544–548.
28. Roff, C. F., E. Goldin, M. E. Comly, A. Cooney, A. Brown, M. T. Vanier, S. P. F. Miller, R. O. Brady, and P. G. Pentchev. 1991. Type C Niemann-Pick disease: use of hydrophobic amines to study defective cholesterol transport. *Dev. Neurosci.* **13:** 315–319.
29. Schimke, R. T. 1988. Gene amplification in cultured cells. *J. Biol. Chem.* **263:** 5989–5992.
30. Stevens, V. L., S. Nimkar, W. C. L. Jamison, D. C. Liotta, and A. H. Merrill, Jr. 1990. Characteristics of the growth inhibition and cytotoxicity of long-chain (sphingoid) bases for Chinese hamster ovary cells: evidence for and involvement of protein kinase C. *Biochim. Biophys. Acta.* **1051:** 37–45.

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 18, 2012