# Reevaluation of the role of the multidrug-resistant P-glycoprotein in cellular cholesterol homeostasis

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**Abstract The multidrug resistance P-glycoprotein (P-gp) was recently proposed to redistribute cholesterol in the plasma membrane, suggesting that P-gp could modulate cholesterol efflux to cholesterol acceptors. To address this hypothesis and to reevaluate the role of P-gp in cholesterol homeostasis, we first analyzed the role of P-gp expression on cholesterol efflux in P-gp stably transfected drug-selected LLC-MDR1 cells. Cholesterol efflux to methyl-β-cyclodextrin (CD) was 4-fold higher in LLC-MDR1 cells compared with control LLC-PK1 cells, indicating that the accessible pool of plasma membrane cholesterol was increased by P-gp expression. However, using the P-gp-inducible cells lines HeLa MDR-Tet and 77.1 MDR-Tet, cholesterol efflux to CD, apolipoprotein A-I, or HDL was not associated with P-gp expression. In addition, we did not observe any effect of P-gp expression on cellular free and esterified cholesterol content, cholesteryl ester uptake from LDL and HDL particles, or acyl-CoA:cholesterol acyltransferase activity. Therefore, we conclude that P-gp expression does not play a major role in cholesterol homeostasis in P-gp-inducible cells and that the effects of P-gp on cholesterol homeostasis previously described in drug-selected cells might result from non-P-gp pathways that were also induced by selection for drug resistance.—**Le Goff, W., M. Settle, D. J. Greene, R. E. Morton, and J. D. Smith. **Reevaluation of the role of the multidrug-resistant P-glycoprotein in cellular cholesterol homeostasis. *J. Lipid Res.* 2006.** 47: **51–58.**

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P-glycoprotein (P-gp) is a member of the ATP binding cassette transporter family responsible for the multidrug resistance (MDR) phenotype. P-gp is the protein product of the human *MDR1* gene (*ABCB1* is the official gene symbol), and P-gp is highly expressed in the intestine, liver, kidney, placenta, and the blood-brain barrier. Plasma membrane P-gp mediates the efflux of numerous neutral and cationic organic compounds and drugs, among them chemotherapeutic drugs, thereby contributing to the MDR phenotype in many cancers. However, information concerning endogenous substrates for P-gp as well as the physiological role of P-gp is still lacking (1).

P-gp has been reported to modulate cellular cholesterol homeostasis via several mechanisms. Stable transfection of a rat intestinal cell line with a P-gp expression vector led to a modest increase in the uptake of cholesterol-containing micelles (2). Nonspecific P-gp inhibitors have been reported to inhibit cholesterol biosynthesis in CHO-7 cells (3) and to inhibit cellular cholesterol esterification (4). Transfection of NIH 3T3 cells with a P-gp expression vector followed by selection for drug resistance was associated with increased esterification of plasma membrane cholesterol (5). Mice, unlike humans, have two copies of the gene for P-gp, *abcb1a* and a*bcb1b* (previously *mdr1a* and *mdr1b*). Mice deficient in both of these genes have been constructed, and although intestinal cholesterol absorption and hepatic free cholesterol (FC) and cholesteryl ester (CE) contents were similar to those of wild-type mice, there were subtle differences in the hepatic uptake of plasma FC and the esterification of oral FC that were not observed in other tissues (6).

To reevaluate the role of P-gp in cellular cholesterol homeostasis, we used three cell systems with variable P-gp expression. We found that drug-selected LLC-MDR1 cells that overexpress human P-pg, versus control cells, had increased FC efflux to methyl-β-cyclodextrin (CD), suggesting that P-gp increased FC cholesterol content in the plasma membrane. However, this effect was not observed in two independent P-gp-inducible cell lines. Nor did we observe any effect of P-gp expression on FC efflux to

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Abbreviations: apoA-I, apolipoprotein A-I; CD, methyl-β-cyclodextrin; CE, cholesteryl ester; COE, cholesteryl oleyl ether; DAPI, 4′,6-diamidino-2-phenylindole; FC, free cholesterol; MDR, multidrug resistance; P-gp, P-glycoprotein; Rho-123, rhodamine 123.

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apolipoprotein A-I (apoA-I) or HDL, cellular FC or CE content, uptake of lipoprotein CE, or ACAT activity. Therefore, P-gp expression does not play a major role in cholesterol homeostasis in P-gp-inducible cells, and the effects on cholesterol homeostasis observed in drug-resistant cells may be attributed to factors other than, or in addition to, P-gp expression.

## EXPERIMENTAL PROCEDURES

### Cell culture

The pig kidney polarized epithelial cell lines LLC-PK1 and its *MDR1* gene-transfected, vincristine-resistant derivative, LLC-MDR1 (7), were kindly provided by Andrei V. Gudkov (Lerner Research Institute, Cleveland Clinic Foundation). LLC-PK1 and LLC-MDR1 were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. HeLa MDR-Tet and 77.1 MDR-Tet cell lines, derived from human HeLa and mouse embryo fibroblasts from *mdr1a/1b* knockout mice, respectively, are stably transfected with human *MDR1* cDNA under the control of a tetracycline-repressed promoter (8) and were a generous gift from Michael M. Gottesman (National Cancer Institute, National Institutes of Health, Bethesda, MD). HeLa MDR-Tet and 77.1 MDR-Tet cells were cultured in high-glucose DMEM with 10% tetracycline-approved FBS (BD Biosciences), 2 mM L-glutamine, and 20 ng/ml colchicine (Sigma). Tetracycline regulation of P-gp expression was performed by a 4 day incubation with colchicine-free medium in the presence ($P\text{-gp}^{Off}$) or absence ($P\text{-gp}^{On}$) of 2 μg/ml tetracycline (Sigma), as described previously (8).

### P-gp activity and protein assays

P-gp activity was assessed by cellular accumulation of the P-gp substrate rhodamine 123 (Rho-123). LLC-PK1 and LLC-MDR1 were plated onto 24-well plates at $2.25 \times 10^5$ per well 1 day before assay. HeLa MDR-Tet and 77.1 MDR-Tet cells were seeded at $8 \times 10^4$ per well onto 24-well plates and incubated in the presence or absence of 2 μg/ml tetracycline for 4 days. Cells were washed twice with PBS and treated with 5 μM Rho-123 with or without verapamil (Sigma) and 2 μg/ml tetracycline when required. After 2 h at 37°C, cells were washed twice with serum-free growth medium and the Rho-123 accumulation was quantified by measuring fluorescence (508/540 nm) at 37 positions in each well (bottom read) using a Spectra Max Gemini EM fluorescent plate reader (Molecular Devices, Sunnyvale, CA). Cells were rinsed twice with PBS, fixed with 10% phosphate-buffered formalin, permeabilized with methanol, and stained with 4′,6-diamidino-2-phenylindole (DAPI). DAPI fluorescence of cell nuclei was quantified by measuring fluorescence (350/470 nm) as described above, and Rho-123 uptake was normalized to DAPI values.

P-gp protein level was assessed by Western blot analysis. Cells were grown on six-well plates in the presence or absence of 2 μg/ml tetracycline for 4 days. The PBS-washed cell pellet was lysed in 100 μl of lysis buffer (2 mM EDTA, 25 mM Tris-phosphate, pH 7.8, 1% Triton X-100, and 10% protease inhibitor cocktail). After discarding the nuclear pellet, the protein concentration was determined using the micro BCA protein assay (Pierce). Analysis of Pg-p protein content was performed by Western blot using 20 μg of cell protein. Blots were incubated sequentially with 1:200 mouse monoclonal antibody raised against human P-gp (p170; Labvision) and 1:10,000 HRP-conjugated goat anti-mouse secondary antibody. The signal was detected with an enhanced chemi-luminescence substrate (Pierce).

### Cholesterol efflux studies

Cells were cholesterol-labeled by incubation for 24 h with 0.5 μCi/ml [$^3$H]cholesterol (Amersham) in DMEM with 1% FBS. Cholesterol efflux to apoA-I (Biodesign, Saco, ME), human HDL, or CD (Sigma) from cholesterol-loaded cells was performed as described previously (9, 10), with specific modifications for different cell lines mentioned in the figure legends. The percentage cholesterol efflux was calculated as 100 × (medium dpm)/(medium dpm + cell dpm).

### Free and esterified cholesterol mass assay

HeLa ($1 \times 10^5$ cells/well) and HeLa MDR-Tet ($8 \times 10^4$ cells/well) cells were plated on 24-well plates and treated with or without 2 μg/ml tetracycline for 4 days. On day 3, cells were washed once with PBS and incubated in either serum-free or 10% FBS-containing medium for 24 h. On day 4, the medium was removed and cellular lipids were extracted in 0.5 ml of hexane-isopropanol (3:2) for 30 min with shaking. The solvent was collected, and the cells were reextracted with an additional 250 μl of hexane-isopropanol. After evaporation of the pooled solvent, lipids were resuspended in 0.5 ml of the reaction buffer (0.1 M potassium phosphate, pH 7.4, 50 mM NaCl, 5 mM cholic acid, and 0.1% Triton X-100) for 5 h with shaking. Fifty microliter aliquots were assayed for FC and CE using the Amplex Red cholesterol assay kit (Molecular Probes) in the presence or absence of cholesterol esterase, respectively. Cells were dissolved in 0.5 ml of 0.1 N NaOH/1% SDS, and a 20 μl aliquot was used for protein determination by the micro BCA protein assay (Pierce). Cholesterol mass values were normalized to cellular protein.

### Cellular uptake of lipoprotein CE

LDL and HDL were isolated by sequential ultracentrifugation and radiolabeled in the presence of [$^3$H]cholesteryl oleyl ether (COE), a nonhydrolyzable analog of CE, via cholesteryl ester transfer protein-mediated transfer from phospholipid/cholesterol liposomes, as described previously (11). HeLa cells ($1 \times 10^5$/well) and HeLa MDR-Tet cells ($8 \times 10^4$/well) were plated onto 24-well plates and treated with or without 2 μg/ml tetracycline for 4 days. On day 3, cells were washed with PBS and incubated in DMEM supplemented with 50 mM glucose, 2 mM glutamine, and 0.2% BSA (DGGB) for 16 h. The uptake of CE was estimated from the uptake of the COE tracer from LDL or HDL in cells incubated in the presence or absence of 2 μg/ml tetracycline as follows: cells were washed once with warm DGGB and incubated with either 10–40 μg/ml [$^3$H]COE-LDL or 15–60 μg/ml [$^3$H]COE-HDL. After 5 h at 37°C, the medium was removed and the cells were washed four times with warm PBS before incubation for a 30 min chase period in DGGB containing 100 μg/ml unlabeled LDL or unlabeled HDL. The cells were then washed four times with warm PBS and dissolved in 0.2 M NaOH. After neutralization with glacial acetic acid, an aliquot of the lysate was counted to determine [$^3$H]COE uptake and assayed for protein determination using the micro BCA protein assay (Pierce). Uptake values were normalized to cellular protein.

### ACAT activity assay

HeLa ($4 \times 10^5$ cells/well) and HeLa MDR-Tet ($3.2 \times 10^5$ cells/well) cells were plated onto six-well plates and treated with or without 2 μg/ml tetracycline for 4 days. Cells were washed once with PBS and incubated with 100 μM oleic acid/BSA complex (8:1 molar ratio) containing a final concentration of 1.25 μCi/ml [9,10(n)-$^3$H]oleic acid (Amersham Biosciences) in DGGB for 1 or 5 h at 37°C. After treatment, the cells were washed three times with PBS, trypsinized, solubilized in 1 ml of PBS,

and sonicated. Seven hundred microliter aliquots were lipid-extracted by the method of Bligh and Dyer (12) and separated by thin-layer chromatography on a K5 silica gel plate (Whatman) developed in a hexane-ethyl ether-acetic acid (70:29:1) solution. Radiolabeled CE, identified by comigration with lipid standards, was scraped from the plate and the dpm were quantified. Aliquots of sonicated cells were used for protein determination by the micro BCA protein assay (Pierce), and radiolabeled lipid values were normalized to cellular protein.

### P-gp localization and cellular FC distribution

HeLa MDR-Tet cells were plated onto poly-lysine-coated four chamber tissue culture glass slides (BD Falcon) at a density of 75,000 cells per chamber and treated with or without 2 μg/ml tetracycline. The cells were grown for 4 days in the selective medium; on the 4th day, the medium was removed and the cells were washed with PBS four times and fixed with 10% buffered formalin for 10 min. The cells were then washed twice with PBS and rocked for 1 h at room temperature in Casein Blocker (Pierce). A 1:200 dilution (in blocker) of mouse monoclonal antibody against P-gp (p170; Labvision) was placed onto the cells for 1 h at room temperature. The antibody was then removed and the cells were washed with PBS five times for 5 min intervals. The secondary antibody, Alexa 594 anti-mouse IgG, was placed onto the cells for 1 h at a 1:500 dilution (in blocker). The antibody was then removed, and the cells were washed with PBS five times for 5 min intervals. Cellular FC distribution was assessed by filipin staining, using the method of Blanchette-Mackie et al. (13). Briefly, a 50 μg/ml filipin solution (Sigma) was freshly prepared from a 10 mg/ml stock in DMSO by dilution with 10% FBS in PBS. The cells were incubated with 1.5 mg/ml glycine in PBS for 10 min at room temperature before staining for 1 h with filipin. The cells were washed with PBS twice and mounted with Vectorshield on a glass coverslip. Using constant settings for both cell treatments, filipin (using a DAPI filter set, but displayed in green) and Alexa 594 (using a Texas Red filter set, displayed in red) fluorescent images were acquired using a 100× oil-immersion lens.

### Statistics

Data are shown as means ± SD. Comparison of two groups was performed by a two-tailed *t*-test, and comparison of three or more groups was performed by ANOVA with Newman-Keuls posttest. All statistical analyses were performed using Prism software (GraphPad, San Diego, CA).

## RESULTS

### P-gp activity and plasma membrane FC in drug-resistant LLC-MDR1 cells

P-gp activity was measured in the pig kidney polarized epithelial cell line LLC-PK1 and in the LLC-MDR1 cell line, which was derived from LLC-PK1 cells by stable transfection with the human *MDR1* gene followed by selection for vincristine resistance (7). As shown in **Fig. 1A**, LLC-MDR1 cells expressed high amounts of human P-gp, whereas no detectable levels were observed in LLC-PK1. P-gp activity was assessed by its ability to pump Rho-123 out of the cells during a 2 h incubation; thus, cellular Rho-123 accumulation is inversely associated with P-gp activity. As shown in Fig. 1B, expression of P-gp in LLC-MDR1 cells significantly decreased cellular Rho-123 accumulation compared with wild-type LLC-PK1 cells (4-fold; $P < 0.001$). Verapamil (100 μM), a competitive inhibitor of P-gp activity, increased Rho-123 accumulation in LLC-MDR1 cells by 93% compared with nontreated cells ($P < 0.01$), and similar results were found with 10 μM verapamil (data not shown). However, this increased level of Rho-123 accumulation was

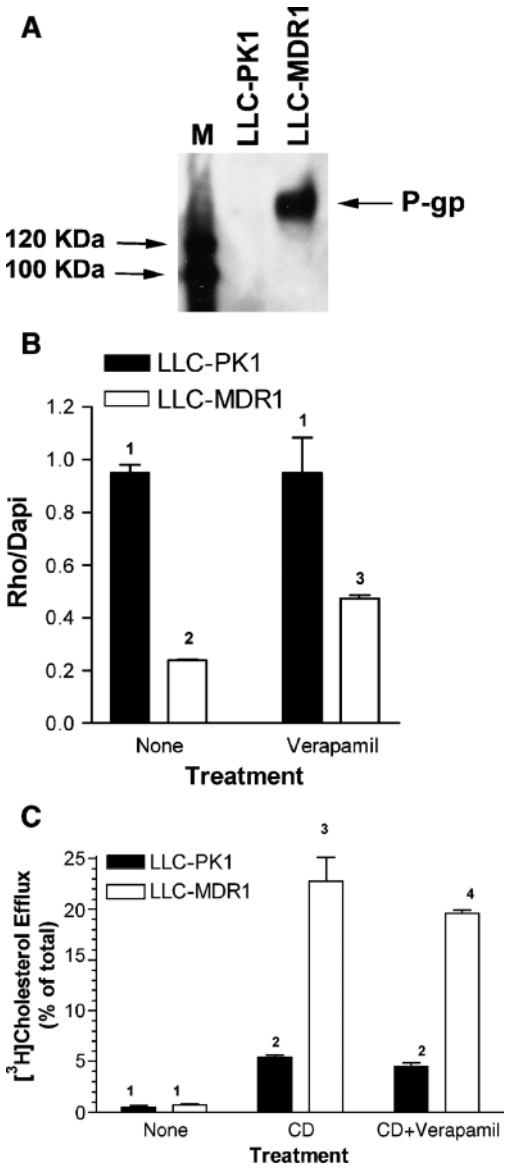


**Fig. 1.** Effects of P-glycoprotein (P-gp) expression on P-gp activity and free cholesterol (FC) efflux to methyl-β-cyclodextrin (CD). A: Western blot analysis indicating that LLC-MDR1 but not LLC-PK1 expressed human P-gp. B: LLC-PK1 (control; closed bars) and LLC-MDR1 (P-gp-expressing; open bars) cells were incubated with 5 μM rhodamine 123 (Rho-123) in the absence or presence of 100 μM verapamil. Intracellular accumulation of Rho-123 uptake normalized to nuclear 4′,6-diamidino-2-phenylindole (DAPI) staining was quantified as an inverse indicator of P-gp activity (n = 3, means ± SD; 1 vs. 2 and 3, $P < 0.001$; 2 vs. 3, $P < 0.01$ by ANOVA). C: FC efflux to 2 mM CD from cholesterol-loaded LLC-PK1 (closed bars) and LLC-MDR1 (open bars) cells in the presence or absence of 100 μM verapamil added during the efflux period. Human P-gp expression increased FC efflux to CD (n = 3, means ± SD; 1 vs. 2, 3, and 4, $P < 0.001$; 2 vs. 3 and 4, $P < 0.001$, 3 vs. 4, $P < 0.01$ by ANOVA).

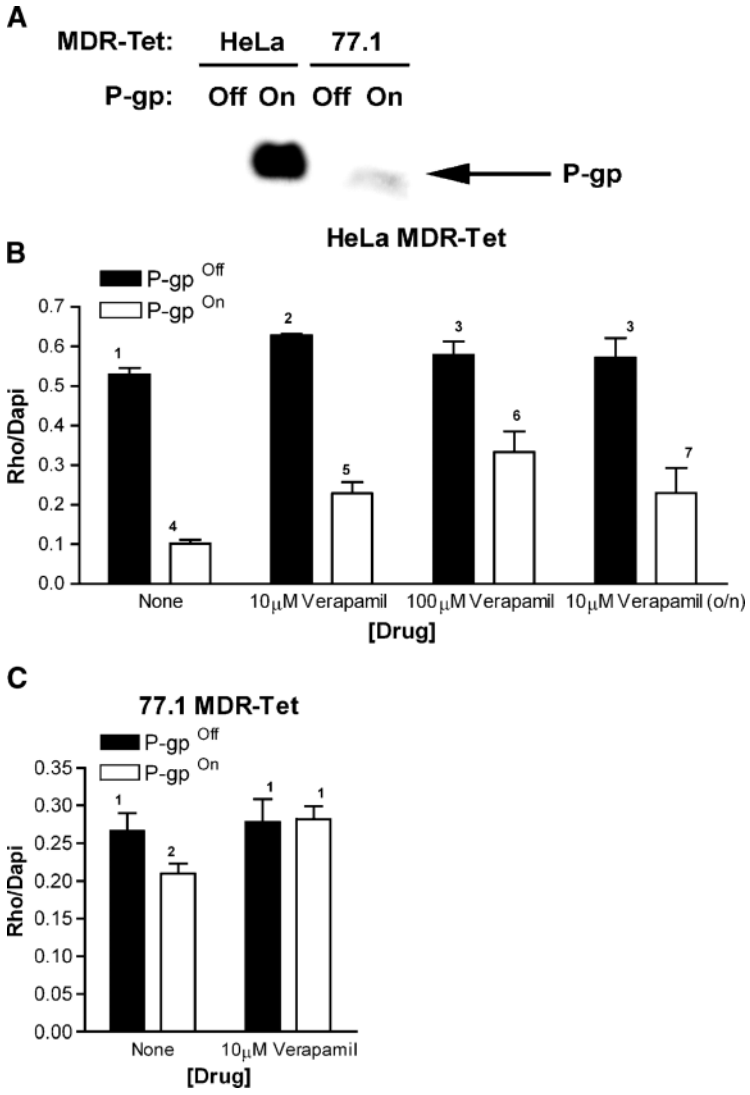


**Fig. 2.** P-gp expression and activity in HeLa MDR-Tet and 77.1 MDR-Tet cells. A: Western blot analysis showing that P-gp protein expression was abolished by a 4 day treatment with 2 μg/ml tetracycline. B, C: P-gp activity in HeLa MDR-Tet (B) and 77.1 MDR-Tet (C) cells in the absence ($P\text{-gp}^{On}$; open bars) or the presence ($P\text{-gp}^{Off}$; closed bars) of 2 μg/ml tetracycline treated with or without verapamil. Intracellular accumulation of Rho-123 normalized to DAPI staining was quantified as an inverse indicator of P-gp activity. P-gp activity was increased in P-gp-expressing cells (n = 3, means ± SD; in B: 1 vs. 4, 5, 6, and 7, $P < 0.001$; 1 vs. 2, $P < 0.05$; 1 vs. 3, NS; 2 vs. 3, NS; 2 vs. 4, 5, 6, and 7, $P < 0.001$; 3 vs. 4, 5, 6, and 7, $P < 0.001$; 4 vs. 5 and 6, $P < 0.001$; 4 vs. 7, $P < 0.01$; 5 vs. 6, $P < 0.01$; 5 vs. 7, NS; 6 vs. 7, $P < 0.01$; in C: 1 vs. 2, $P < 0.05$ by ANOVA).

still <50% of the level observed in control LLC-PK1 cells ($P < 0.001$), and, as expected, verapamil had no effect on Rho-123 accumulation in LLC-PK1 cells. Thus, the decreased Rho-123 accumulation in LLC-MDR1 cells confirmed their increased P-gp activity; however, the only partial recovery of Rho-123 accumulation with verapamil treatment implies that other pathways (not sensitive to verapamil inhibition) may differ between LLC-MDR1 cells and the control LLC-PK1 cells.

If P-gp alters the plasma membrane FC pool or its distribution, we would expect this to be reflected in FC release to extracellular acceptors. We analyzed FC efflux from these cell lines to CD during a 10 min incubation. We and others have previously used FC efflux to CD, in moderate doses for short time periods, as an indicator of plasma membrane FC content (10, 14). FC efflux to CD from LLC-MDR1 cells was 4-fold higher than that observed from LLC-PK1 ($P < 0.001$; Fig. 1C), an increase consistent with the increase of P-gp activity observed in LLC-MDR1 cells (Fig. 1B). Incubation with 100 μM verapamil reduced FC efflux to CD from LLC-MDR1 cells moderately by 17% ($P < 0.01$), without affecting efflux from LLC-PK1 cells. Together, our experiments in drug-selected LLC-MDR1 cells suggest that expression of human P-gp increases the FC content accessible to CD in plasma membrane, thus supporting a role of P-gp in cellular cholesterol homeostasis. However, these observations must be interpreted with caution, because these experiments were performed with cells that were chronically selected for vincristine resistance; thus, the effects on plasma membrane FC may be mediated by non-P-gp pathways that were also induced by selection for drug resistance.

### P-gp activity and plasma membrane FC in cells with P-gp expression driven by a tetracycline-repressible promoter

To reexamine the role of P-gp in cellular cholesterol homeostasis without the complications caused by using cells selected for drug resistance, we used two cells lines, HeLa MDR-Tet and 77.1 MDR-Tet, generated in the laboratory of Michael M. Gottesman (8). These cell lines were made by stable cotransfection of the human *MDR1* cDNA driven by a tetracycline-repressible promoter along with the expression vector for the tetracycline-responsive repressor into HeLa cells and mouse embryonic fibroblasts

from *mdr1a/1b* doubly deficient embryos, respectively. Thus, P-gp is turned off in the presence of tetracycline (P-gp$^{Off}$) and induced in the absence of tetracycline (P-gp$^{On}$). We confirmed by Western blot that tetracycline treatment completely abolished human P-gp expression in both cell lines, with the level of P-gp substantially higher in HeLa MDR-Tet cells than in 77.1 MDR-Tet cells in the P-gp$^{On}$ state (**Fig. 2A**).

P-gp activity in both of these cell lines was assessed by Rho-123 accumulation. Congruent with previously published P-gp activity assays for these cell lines (8), expression of human P-gp significantly reduced Rho-123 accumulation in both HeLa MDR-Tet and 77.1 MDR-Tet cells ($P < 0.001$ and $P < 0.05$, respectively; Fig. 2B, C). Consistent with the lower P-gp protein levels in 77.1 P-gp$^{On}$ cells compared with HeLa P-gp$^{On}$ cells, Rho-123 accumulation was diminished 4-fold in HeLa P-gp$^{On}$ cells (vs. HeLa P-gp$^{Off}$) and only 1.3-fold in 77.1 P-gp$^{On}$ cells (vs. 77.1 P-gp$^{Off}$), reflecting a higher P-gp activity in HeLa P-gp$^{On}$ cells than in 77.1 P-gp$^{On}$ cells. Inhibition of P-gp activity by 10 μM verapamil led to a 124% increase in Rho-123 accumulation in HeLa P-gp$^{On}$ ($P < 0.001$), but still did not achieve the level of Rho-123 accumulation in the HeLa P-gp$^{Off}$ cells, and a 19% increase in HeLa P-gp$^{Off}$ cells ($P < 0.001$). A higher concentration of verapamil (100 μM) only slightly further increased Rho-123 accumulation in HeLa P-gp$^{On}$ (+46% vs. 10 μM verapamil; $P < 0.01$), whereas an overnight incubation with 10 μM verapamil had no significant further effect (Fig. 2B).

Thus, verapamil had both P-gp-dependent and -independent effects in the HeLa MDR-Tet cell line, and verapamil could not fully inhibit P-gp activity in these cells with very high expression of P-gp. Verapamil led to a 34% increase in Rho-123 accumulation in the 77.1 P-gp$^{On}$ cells ($P < 0.05$), restoring the Rho-123 accumulation level to that observed in the 77.1 P-gp$^{Off}$ cells; also, verapamil had no effect on Rho-123 accumulation in these 77.1 P-gp$^{Off}$ cells (Fig. 2C). Thus, verapamil had only P-gp-dependent effects in this cell line, and verapamil could fully inhibit P-gp activity in these cells with very modest expression of P-gp.

We analyzed the capacity of HeLa MDR-Tet and 77.1 MDR-Tet cells to mediate FC efflux to CD and to the other exogenous acceptors apoA-I and HDL. The expression versus lack of expression of P-gp had no significant effect on FC efflux to CD, apoA-I, or HDL in both HeLa MDR-Tet and 77.1 MDR-Tet cell lines (**Fig. 3A**, **B**). ABCA1 induction via a 16 h incubation with 4 μg/ml 22-hydroxycholesterol plus 1 μM 9-*cis* retinoic acid increased FC efflux to apoA-I from both cell lines, but again, expression of P-gp did not alter this induced efflux within either cell line (data not shown). Time course analysis of FC efflux to CD confirmed the absence of a P-gp effect in both cell lines, even at the earliest time points, indicative that P-gp expression had no effect on FC levels in the plasma

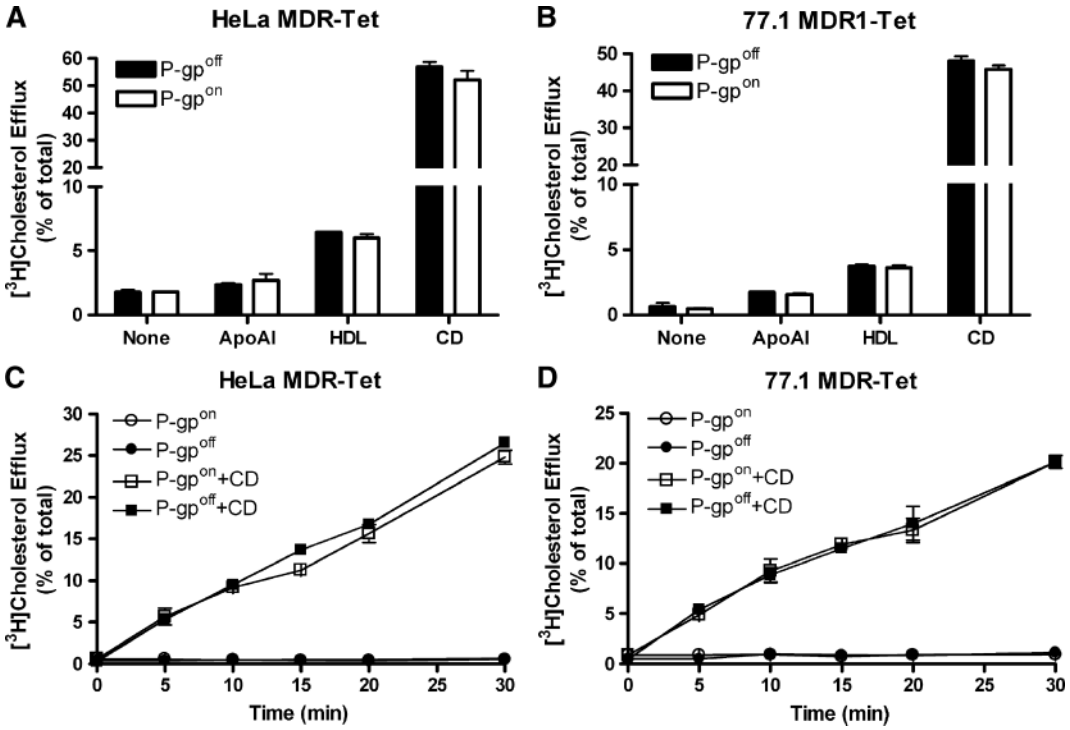


**Fig. 3.** Lack of effect of P-gp expression on cholesterol efflux to extracellular acceptors from HeLa MDR-Tet and 77.1 MDR-Tet cells. A, B: FC efflux from HeLa MDR-Tet (A) or 77.1 MDR-Tet (B) cells to 5 mM CD for 30 min at 37°C or 3 μg/ml apolipoprotein A-I (apoA-I) or 30 μg/ml HDL for 4 h at 37°C in the presence (P-gp$^{On}$; open bars) or the absence (P-gp$^{Off}$; closed bars) of P-gp expression (n = 3, means ± SD). C, D: Time course of FC efflux to 1 mM CD (squares) or medium alone (circles) from HeLa MDR-Tet (C) and 77.1 MDR-Tet (D) cells in the absence (closed symbols) or the presence (open symbols) of P-gp expression (n = 3, means ± SD).

membrane (Fig. 3C, D). Thus, these results did not confirm the P-gp association with FC levels in the plasma membrane observed in the drug-selected LLC-MDR1 cells.

### Cholesterol metabolism in cells with P-gp expression driven by a tetracycline-repressible promoter

We measured total cholesterol, FC, and CE in HeLa MDR-Tet cells maintained in culture medium containing 10% FBS, and we found no effect of P-gp expression on these parameters (data not shown). We repeated this experiment in these cells that had been deprived of FBS for 24 h, and although CE was decreased to barely detectable levels, there was still no effect of P-gp expression on total cholesterol, FC, or CE level (data not shown). Cellular FC, as detected by filipin staining, was distributed in the plasma membrane and in perinuclear vesicles, and this distribution was not qualitatively altered in HeLa cells by the presence or absence of MDR expression in many independent cell preparations (**Fig. 4A**, **C**). P-gp staining was observed only in the Tet P-gp$^{On}$ cells, and it was also distributed in the plasma membrane and in intracellular vesicles (Fig. 4B, D). Although there was partial overlap of MDR and cholesterol in some intracellular vesicles, there were also vesicular regions that were enriched for only cholesterol or MDR (Fig. 4C–E). Cholesterol mass assays and filipin staining experiments were also performed in wild-type HeLa cells, and we found no effects of tetracycline treatment on any of these parameters (data not shown). Thus, modulation of P-gp expression did not alter cellular cholesterol levels or FC distribution in HeLa MDR-Tet cells.

The dose-dependent uptake of LDL- and HDL-incorporated COE, a nonmetabolized tracer for CE, was measured during a 5 h incubation at 37°C in HeLa MDR-Tet cells that were preincubated for 16 h in serum-free medium. COE uptake from LDL and HDL was dose-dependent; however, P-gp expression had no effect on COE uptake from either LDL or HDL (data not shown). Similar results were observed in wild-type HeLa cells treated with or without tetracycline (data not shown). Thus, P-gp expression did not affect uptake of the CE tracer from LDL or HDL.

Finally, the cellular activity of ACAT to convert FC into CE was estimated in HeLa MDR-Tet cells by incubation with [$^3$H]oleic acid in serum-free medium for 1 and 5 h at 37°C. P-gp expression had no effect on ACAT activity at either time point (data not shown). Similar results were observed in wild-type HeLa cells treated with or without tetracycline (data not shown). Thus, cellular ACAT activity was not altered by the expression of P-gp in HeLa MDR-Tet cells.

## DISCUSSION

Several studies performed in drug-selected cells report that P-gp plays a role in cholesterol homeostasis. Using membrane vesicles prepared from insect cells infected with a P-gp encoding baculovirus leading to a high level of expression, P-gp was reported to increase membrane cholesterol accessibility to cholesterol oxidase (15), which the authors suggest to be the result of P-gp activity as a cholesterol translocase. However, this result might instead be attributable to the overexpression of P-gp, which has been demonstrated to alter liposome lipid packing (16), which could lead to a subsequent effect on cholesterol oxidase accessibility to membrane cholesterol.

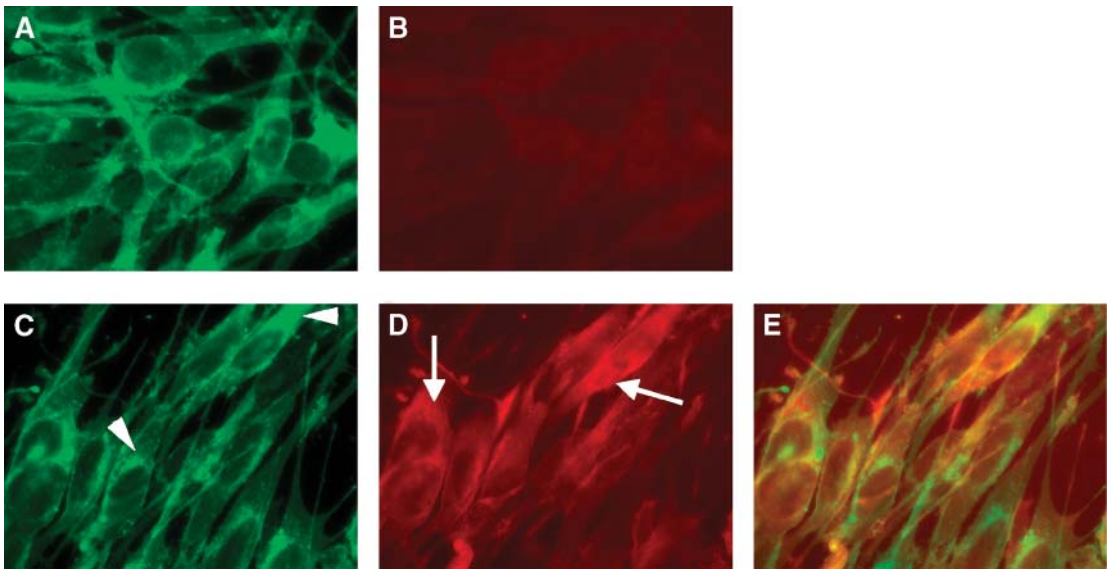


**Fig. 4.** Lack of effect of P-gp expression on intracellular cholesterol distribution in HeLa MDR-Tet cells. A, B: Cells grown in the presence of 2 μg/ml tetracycline for 4 days to extinguish multidrug resistance (MDR) expression were doubly stained with filipin to detect FC (A) and with an antibody to detect human P-gp (B). C–E: Cells grown in the absence of tetracycline to allow MDR expression were doubly stained with filipin to detect FC (C) and with an antibody to detect human P-gp (D); the merged image is also shown (E). The arrowheads in C show perinuclear vesicular cholesterol deposits that are not particularly enriched in MDR expression. The arrows in D show perinuclear vesicular MDR deposits that are not particularly enriched for cholesterol.

We first examined the effect of P-gp expression on plasma membrane cholesterol in drug-selected LLC-MDR1, and we found that P-gp expression was associated with an increase in cholesterol efflux to CD, suggesting that P-gp expression increased the accessible pool of plasma membrane cholesterol. However, we then found that P-gp expression did not affect cholesterol efflux to CD in P-gp-inducible cells. We also observed that FC efflux to apoA-I or HDL, and the uptake of CE from HDL or LDL, were not associated with the expression of P-gp in the inducible cell lines. Together, these data suggest that most of the effects on cholesterol homeostasis observed in some drug-resistant cell lines might be attributable to effectors other than P-gp that are altered by the selection for drug-resistant cells. A similar discrepancy in results obtained with drug-selected cells versus P-gp-inducible cells has been reported previously (8). Several drug-selected cells expressing P-gp were found to have increased membrane fluidity and membrane potential, whereas these effects were not observed in the P-gp-inducible HeLa MDR-Tet and 77.1 MDR-Tet cells (8). Thus, it appears that some of the findings made using drug-selected cells may result from drug selection rather than from P-gp expression.

Other studies have focused on the role of P-gp in cholesterol transport from the plasma membrane to the endoplasmic reticulum by assaying the esterification of plasma membrane-derived cholesterol by ACAT, which is localized in the endoplasmic reticulum. For example, verapamil, a nonspecific P-gp inhibitor, was found to inhibit cellular cholesterol esterification (4, 5), and P-gp-transfected NIH 3T3 fibroblasts grown under drug selection were also found to have increased cholesterol esterification, without an increase in plasma membrane cholesterol content (5). However, another report, although repeating the verapamil effect, failed to find any effect of a specific P-gp inhibitor, GF120981, on the esterification of lipoprotein-derived cholesterol in HepG2 cells (17). Our study in a P-gp-inducible cell line also failed to find any effect of P-gp expression on cellular cholesterol esterification or on the mass ratio of cellular CE to FC. Finally, data from the *mdr1a/1b* double knockout mouse show that cholesterol esterification, compared with wild-type mice, is normal in all tissues except for the liver, in which an oral cholesterol bolus is esterified to a greater extent at the 6 h time point (but not at 24 or 72 h), whereas an intravenous cholesterol bolus is esterified to the same extent in the liver of both types of mice (6). Thus, P-gp does not appear to play a role in cholesterol esterification in most tissues, although P-gp may play a minor role in the liver.

Previously, cholesterol was also found to play a regulatory role in P-gp expression (18) and activity (15, 16, 19). The current study did not address these issues. However, the observed effects of cholesterol depletion on P-gp ATPase activity in membrane vesicles (15) could also be the result of nonspecific effects on membrane fluidity and lipid packing (16).

Finally, P-gp-inducible cells represent a very useful model in which to study the role of P-gp to overcome limitations resulting from the use of drug-selected cells or P-gp inhibitors. Verapamil and cyclosporine A, two molecules known to inhibit P-gp activity, were recently shown to modulate ABCA1 transporter activity (20, 21), a key protein in cholesterol homeostasis. The reevaluation of the role of P-gp in cholesterol homeostasis using P-gp-inducible cells has led us to conclude that P-gp does not play a major role in cholesterol homeostasis.

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