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# P-Glycoprotein, Expressed in Multidrug Resistant Cells, Is Not Responsible for Alterations in Membrane Fluidity or Membrane Potential[1]

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## ABSTRACT

**Expression of P-glycoprotein (P-gp), the *multidrug resistance (MDR) 1* gene product, can lead to MDR in tumors. However, the physiological role of P-gp in normal tissues is not well understood. Previous studies on multidrug-resistant cells have suggested changes in membrane fluidity and membrane potential associated with P-gp expression, but interpretation of these studies is difficult, because most experimental cells have been selected for long periods in the presence of cytotoxic drugs and may have other host alterations. Therefore, we created two cell lines in which a transfected human *MDR1* cDNA is repressed by tetracycline and induced in the absence of tetracycline. One cell line was derived from a mouse embryonic fibroblast cultured from a double (*mdr*1a/1b) knockout mouse, and the other was from a human HeLa cell line. Analysis of the kinetics of expression of P-gp showed that the mRNA had a half-life of ~4 h, and the protein had a half-life of ~16 h. P-gp cell surface expression (measured with monoclonal antibody MRK-16) and P-gp function (measured with a fluorescent substrate, rhodamine 123) was characterized by using fluorescence-activated cell sorting. No differences in membrane potential using the fluorescent probe oxonol or in membrane "fluidity" using fluorescent anisotropy probe or electron spin resonance probe were observed in the tet-repressible P-gp-expressing cells. In contrast, several drug-selected cells that express P-gp showed an increase in membrane fluidity and membrane potential. These results suggest that expression of P-gp *per se* has little effect on membrane fluidity or membrane potential, and it does not have $H^+$ pump activity. The changes in these parameters observed in drug-selected cells must reflect other host adaptations to drug selection.**

## INTRODUCTION

P-gp[3] belongs to the class of ABC transporters (ABCB1) and is the protein product of the *MDR*1 gene. It is a drug transporter with broad specificity for many amphipathic, organic substrates (1–3). In humans, P-gp is constitutively expressed in the intestine, liver, kidney, placenta, and the blood brain barrier (4). It has been shown to have a protective effect against xenobiotics that enter the body through the intestines or the brain through the blood brain barrier (5). The complete physiological role and the endogenous substrates of P-gp are not known. Mice lacking *mdr*1a and *mdr*1b have normal life spans in the laboratory under protective care (5). In many cancers, P-gp is overexpressed contributing to resistance to clinically important chemotherapeutic drugs that are P-gp substrates, including doxorubicin, vinblastine, and Taxol.

In the laboratory, many different cell lines have been used to demonstrate effects of P-gp on the plasma membrane, on drug resistance, on apoptosis, and on resistance to viruses in an effort to elucidate the physiological role of P-gp (6–12). It is not surprising that results obtained from different cell lines and expression systems are not consistent with each other. The question arises whether the results obtained are because of the selection pressure on the cells, or other unknown phenomena that occur in cells that have been in culture, in some cases, for a number of years.

We have developed one mouse and one human cell line in which human *MDR*1 is under tetracycline control. In these cells, addition of tetracycline rapidly turns off transcription of *MDR*1 mRNA, and over a period of a few days P-gp disappears from the cells, allowing a comparison of the same cells with and without P-gp in the plasma membrane. In this study, we characterize the P-gp mRNA half-life, P-gp protein half-life, and measure membrane properties, such as potential and fluidity.

To assess plasma membrane biophysical characteristics, such as fluidity, of normal and tumor cells, ESR was used. ESR was used previously to measure membrane fluidity of transplanted melanoma cells (13), human lung tumor and normal tissues (14), and cisplatin-sensitive and -resistant cells (15). Also, high and low metastatic Lewis lung cancer cells were shown to have different plasma membrane fluidity by using ESR (16). Fluidity of plasma membranes was also studied using lipid soluble fluorescence probes, such as DPH (15). Wanten and Naber (17) used DPH and TMA-DPH in their studies, which we used in our investigation here. Measurement of plasma membrane potential is indicative of the general biological status of cells. For example, alteration of membrane connected proteins changes membrane potential (18, 19), and antiproliferative signals induce plasma membrane depolarization in lymphocytes (20). Using these techniques, we show that drug-selected cells frequently show changes in membrane properties, whereas P-gp expression alone does not affect these properties.

## MATERIALS AND METHODS

**Insertion of MDR1 cDNA into Tet-repressible Vectors.** The plasmids, pRevTRE and pRevTet-Off were purchased from Clontech (CLONTECH Laboratories, Inc., Palo Alto, CA). The human *MDR*1 gene was cloned into *BamH*1 and *Cla*1 sites in the multiple cloning site of the pRevTRE plasmid. The sequence was verified using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) in a Perkin-Elmer GeneAmp System 9600. The conditions were: 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. A series of six primers were used to sequence the entire insert. The forward direction primers used were MDR516 (5′: CAC-CCG-ACT-TAC-AGA-TGA), MDR1159 (5′: AAT-ATT-AAG-GGA-AAT-TTG-GAA), and MDR3447 (5′: AAA-GGA-GGC-CAA-CAT-ACA). The reverse direction primers used were MDR964R (5′: AGA-ATA-TTC-CCC-TGA-GAG), MDR1340R (5′: CAC-TGA-CCA-TCC-CCT-CTG), and MDR3747R (5′: GTG-CCA-TGC-TCC-TTG-ACT-CTG). The reactions were column purified with Auto Seq G-50 to eliminate the excess primers (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The reactions were electrophoresed in the National Cancer Institute Sequencing Core Facility. After

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[3] The abbreviations used are: P-gp, P-glycoprotein; MDR, multidrug resistance; ESR, electron spin resonance; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA, trimethyl-ammonium; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; RT, reverse transcription-PCR; Cp, crossing point; AOSA, 6-(9-anthroyloxy)stearic acid; T-SASL, 2,2,6,6-tetramethyl piperidin-1-oxyl-4-yl-octadecenoate; 5-doxyl-SA, 5-doxyl stearic acid; mAb, monoclonal antibody.

verification that the sequences were correct, the plasmids were grown in large scale and purified using the CsCl method (Lofstrand Labs Limited, Gaithersburg, MD).

**Cell Lines and Cell Culture.** All of the Tet-Off cells were cultured in DMEM high glucose (Life Technologies, Inc.) with 10% tetracycline-approved FBS (Clontech), and 2 mM L-glutamine (Quality Biological, Inc.) at 37°C with 5% $CO_2$. HeLa Tet-Off cells (Clontech) are the parental cells of HeLa MDR-Off. HeLa P-gp$^{on}$ cells refer to HeLa MDR-Off cells that were grown without colchicine for at least 4 days. HeLa P-gp$^{off}$ cells refer to HeLa MDR-Off cells that were grown in the presence of 2 $\mu$g/ml tetracycline and are not expressing P-gp. The nondrug-selected cells KB-3-1 (a subclone of HeLa), and drug-selected KB-8-5, KB-C1, KB-V1, KB-A1, and KB-A1rev (21, 22) were used in this study. Cells selected with colchicine are KB-8-5 (22) and KB-C1 (21). Cells selected with vinblastine are KB-V1, and cells selected with doxorubicin are KB-A1. KB-A1rev cells are KB-A1 grown without drug selection, have lost their resistance, and are, thus, considered revertants. 77.1 cells were a generous gift from Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) and are mouse embryo fibroblasts derived from mdr1a/1b knockout mice (5) used to make the 77.1 MDR-Off cell line. 77 P-gp$^{on}$ refers to 77.1 MDR-Off cells grown without colchicine for at least 4 days. 77 P-gp$^{off}$ refers to 77.1 MDR-Off cells grown in the presence of 2 $\mu$g/ml tetracycline for 4 days.

**Stable Transfection.** The plasmid containing the human *MDR*1 gene, pRevTRE-MDR1 and the plasmid containing the transactivator, pRevTet-Off, were cotransfected into 77.1 cells. The pRevTRE-MDR1 plasmid was transfected into HeLa Tet-Off cells that are already stably transfected with pRevTet-Off. Using the LipofectAMINE PLUS system (Life Technologies, Inc., Rockville, MD), HeLa Tet-Off cells were incubated with 4 $\mu$g pRevTRE-MDR1, 20 $\mu$l PLUS reagent and 30 $\mu$l LipofectAMINE for 3 h in 100-mm dishes (Falcon). 77.1 cells were incubated with 4 $\mu$g pRevTRE-MDR1 and 4 $\mu$g pREVTet-Off along with the LipofectAMINE and PLUS reagent as described above. The cells were then washed and were allowed to recover overnight in DMEM supplemented with 10% tetracycline-approved FBS (Clontech) and 2 mM L-glutamine. The next day, 20 ng/ml colchicine was added to the medium for selection. Ten days later, colonies were picked and transferred to 12-well plates. Seven days later, the cells were transferred to T25 flasks, and assayed for the expression and function of P-gp.

**P-gp Membrane Expression FACS Assay.** Cells were treated with 2 $\mu$g/ml tetracycline for 0–4 days. Subsequently, these treated cells were trypsinized daily from their flasks, harvested, and washed in Iscove's Modifed Dulbecco's Medium supplemented with 5% FBS. Cells were then resuspended in 200 $\mu$l of IMDM/5% FBS containing 5 $\mu$g of MRK16, a mAb specific for surface epitopes of human P-gp. After initial incubation at 37°C for 20 min the MRK16-treated cells were washed and then incubated with a secondary antibody, FITC antimouse $IgG_{2a}$ (PharMingen), for 20 min. The antibody-treated cells were washed and then analyzed on a FACSCalibur (Becton Dickinson), operated with a 15 mW argon ion laser tuned to 488 nm excitation wavelength. Fluorescence emission was collected at 525 nm. Controls used in the FACS analysis were cells that had not been incubated with antibody, cells that had been incubated with an isotype standard mouse $IgG_{2a}$ followed by FITC antimouse $IgG_{2a}$, and cells incubated with FITC antimouse $IgG_{2a}$ alone. Control profiles were similar to cells alone without any antibody incubation. Triplicate samples were assayed, and their histograms were analyzed. The median fluorescence was plotted on a log scale, and the half-life of the protein was calculated by using the equation: $y = 100e^{-0.0442x}$ where y is percentage of expression and x is time in tetracycline. $R^2 = 0.9879$ for this equation.

**P-gp Function FACS Assay.** Cells were treated with tetracycline and trypsinized from the flasks as described above, and were resuspended in 5 ml IMEM with 5 $\mu$M cyclosporin A for 10 min at 37°C. Rhodamine 123 was added to a final concentration of 0.5 $\mu$g/ml to cells both treated and untreated with cyclosporin A for 20 min at 37°C. Cells were then washed and analyzed on a FACSCalibur (Becton Dickinson). Controls were cells not incubated with cyclosporin A or rhodamine 123. Triplicate samples were measured, and the histograms were analyzed. The median fluorescence was plotted against the time in tetracycline on a log scale. The data were presented as accumulation of rhodamine 123, which directly reflects the efflux of the substrate and, thus, the function of P-gp.

**Purification of RNA.** Five $\times 10^6$ cells treated with or without tetracycline were centrifuged at 1300 rpm, 4°C, for 5 min. The pellet was stored at −70°C. The Qiagen RNeasy kit was used (Qiagen, Valencia, CA) to extract RNA. The cells were lysed and homogenized with a 20-gauge syringe in the lysis buffer provided. RNA purity was confirmed by a 260 nm:280 nm ratio of 2.1 on a Pharmacia Biotech Ultraspec 3000 in 1× 10 mM Tris (pH 8)-1 mM EDTA buffer (Cambridge, United Kingdom). RNA was then electrophoresed on 1.25% agarose/formaldehyde gel. RNA was stored at −70°C at a 1 $\mu$g/$\mu$l concentration.

**Western Blot.** Frozen cell pellets from cells treated with or without tetracycline were thawed and dissolved in a lysis buffer containing 10 mM Tris, 0.1% Triton X-100, 10 mM $MgSO_4$, 2 mM $CaCl_2$, 1% aprotinin, 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 2 mM DTT, and 20 $\mu$g/ml DNase. Samples were incubated at 37°C for 5 min and then frozen in dry ice. Samples were then thawed and sonicated. Cell lysates were mixed with Novex Tris-Glycine SDS sample buffer (Invitrogen) and incubated at 37°C for 30 min. Protein was quantitated using a BCA assay (Pierce). Three $\mu$g of protein was loaded on a Novex 4–20% Tris-glycine gel (Invitrogen). Protein was transferred to a 0.45 $\mu$m pore size nitrocellulose membrane for 4 h at 200 milliAmps using a Mini-PROTEAN II Cell apparatus (Bio-Rad). Membranes were then blocked overnight in a 20% milk, 0.5% Tween 20, PBS buffer. Membranes were washed with 0.5% Tween 20, PBS buffer, and then incubated with the C219 primary antibody (Fujirebio Diagnostics, Inc.). C219 is a mAb that reacts with an internal membrane epitope of human P-gp, and 6C5 is a mAb against rabbit GAPDH (Research Diagnostics, Inc). A 1:2000 dilution of C219 and 1 $\mu$g/ml 6C5 were added to a 5% milk, 0.5% Tween 20, PBS buffer, and incubated with the membrane at room temperature for 2 h on a shaker. The membrane was then washed in the 0.5% Tween 20, PBS buffer. The secondary antibody is a peroxidase-labeled mouse IgG (KPL). A 1:5000 dilution of the secondary antibody was made into a 5% milk, 0.5% Tween 20, PBS solution and incubated with the membrane for 45 min at room temperature on a shaker. Enhanced chemiluminescence was performed as directed by the manufacturer (Amersham Pharmacia Biotech).

**Real-Time PCR.** Real-time PCR was performed with a Light Cycler DNA SYBR Green kit (Roche Biochemicals, Indianapolis, IN). The reaction was in a 20 $\mu$l final volume with 0.5 $\mu$g of purified total RNA, 4 $\mu$l PCR mix provided by the manufacturer, 4 $\mu$l $MgCl_2$ (25 mM), 1 $\mu$l of each primer (10 $\mu$M), 0.4 $\mu$l of enzyme mix and DEPC-$H_2O$. Primer sequences for the genes amplified were as follows: ABCB1 (5′-GGG-ACC-GCA-ATG-GAG-GAG and 5′-GCA-CCA-ATT-CCA-CTG-TAA), ABCB1-UTR-(5′-TGA-CAT-T-TA-TTC-AAA-GTT-AAA-AGC-A and 5′-TAG-ACA-CTT-TAT-GCA-AA-C-ATT-TCA-A), and GAPDH (5′-CCC-TTC-ATT-GAC-CTC-AAC-TAC-AT and 5′-ACG-ATA-CCA-AAG-TTG-TCA-TGG-AT). The RT reaction was performed at 55°C for 20 min. cDNA generated by the RT step was denatured at 95°C for 20 s. Amplification of the cDNA was done in 45 cycles of 95°C for 5 s, 58°C for 10 s, and 72°C for 13 s. Fluorescence was recorded during the elongation phase at 72°C. Determination of the number of cycles it took to see a PCR product or Crossing point (Cp) was performed on the basis of the second derivative algorithm. As a control for quantitative PCR, a standard was made with increasing copy number of pRevTRE- MDR1 plasmid from $10^5$ to $10^{10}$ copies to calculate the number of cycles needed to double the amount of RNA. The equation used was $Cp = 3.9 \log_{10}(\text{copy number}) + 49.08$. The number of cycles calculated was 1.17 to double the amount of RNA in the real time RT-PCR assay. Real-time PCR of endogenous MDR1 and total MDR1 was done on the tetracycline time course samples, and they were all normalized to GAPDH. $\Delta$Cp are Cp values that have been normalized to GAPDH. The deviation of $\Delta$Cp of certain time points (t) from the $\Delta$Cp when time of tetracycline is zero ($t = 0$) was calculated and plotted against the time of tetracycline treatment.

**Drug Resistance Assay.** Colony formation assays were used to determine the colchicine, doxorubicin, and vinblastine sensitivity of the HeLa MDR-Off cell line relative to the parental cell line, HeLa Tet-Off, after a 4-day incubation in the presence or absence of 2 $\mu$g/ml tetracycline. After their initial 37°C incubation in T25 flasks, the monolayers were trypsinized, and a Coulter Counter was used to establish the total number of cells per flask. Three hundred exponentially growing cells from each flask were seeded into 60-mm dishes containing DMEM ± 2 $\mu$g/ml of tetracycline. The cells were allowed to attach for ~16 h before increasing concentrations (*i.e.*, 0, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1,000, 3,000, 10,000, and 30,000 ng/ml) of colchicine, doxorubicin, or vinblastine were added to the existing medium. Dose response curves were generated by allowing the cells to form colonies over an additional

8–10-day incubation before staining with 0.5% methylene blue in 50% ethanol and counting them with an IS-1000 Digital Imaging System. For each cell line, the percentage of the relative cloning efficiency per drug concentration was determined by dividing the number of colonies counted after drug treatment by the number of colonies formed with zero drug treatment. Percentage points were plotted to determine the $LD_{10}$ drug concentration, which reduced the cloning efficiency of the transfectants to 10% of the control without drug. Relative resistance was determined by dividing the $LD_{10}$ value of the resistant line by the $LD_{10}$ value of the parental line.

**Membrane Potential Measurements.** Membrane potential measurements were done by flow cytometry using the negatively charged $DiBaC_4$ (3) oxonol dye (Molecular Probes, Eugene, OR), essentially as described earlier (23). Briefly, a cell suspension of $1 \times 10^6$ cells/ml was equilibrated for 1 min in PBS followed by 150 nM oxonol dye. After a 2-min equilibration at room temperature, data were collected and displayed as histograms from $10^4$ cells/sample. Reproducibility was determined by measuring membrane potential of the cells on different days from separate cultures. Oxonol fluorescence intensity measures membrane potential when the extracellular potassium concentration is changed from 5 mM to 150 mM, resulting in an increase of the fluorescence intensity of the oxonol stained cells because of their depolarization. All of the measurements were made with a Becton Dickinson FacsCalibur flow cytometer (Becton Dickinson, Mountain View, CA), operated with a 15-mW argon ion laser tuned to 488 nm excitation wavelength. Fluorescence emission was collected at 525 nm. Results are expressed in comparative histograms of a representative series of cells.

**Polarity of Fluorescent Membrane Probes.** Measurements of steady-state fluorescence polarization were done with a spectrofluorometer LS50B (Perkin-Elmer, Norwalk, CT) and the lipid soluble fluorophore, TMA-DPH (Molecular Probes). This fluorophore is known to probe membranes at the surface. TMA-DPH was dissolved in tetrahydrofurane at a concentration of 2 mM and was kept in the dark at 4°C. Cells ($10^6$/ml) were labeled with TMA-DPH at a concentration of 2 $\mu$M in PBS. After a 10-min incubation at 4°C, the cell suspension was centrifuged and washed twice in the centrifuge with PBS. After resuspension in PBS, fluorescence anisotropy was measured at 30°C. The excitation wavelength was 355 nm, and the emission was measured at 430 nm with a slit width of 5. The polarization was calculated according to Collins *et al.* (24) by the equation $P = (I_{0,0} - G*I_{0,90})/(I_{0,0} + G*I_{0,90})$ from the measured fluorescence intensities. Another polarization probe, AOSA, (Molecular Probes), was also used to measure polarization of cells. This probe can assess membrane fluidity six carbons deep in the plasma membrane, whereas TMA-DPH probes at the surface of the plasma membrane. The same experimental conditions were used with this polarization probe as with TMA-DPH, given above, except that 360 nm excitation and 470 nm emission wavelengths were used.

**ESR Studies on P-gp Expressing and Nonexpressing Cells.** ESR studies were conducted with 5-doxyl-SA and with T-SASL probes (Molecular Probes). The 5-doxyl-SA probe intercalates to a depth of five carbons in the outer leaflet of the plasma membrane (25), and T-SASL probes the surface of the plasma membranes (26). Labeling the cells with the spin probes was done as follows: 1 mg/ml 5-doxyl-SA dissolved in ethanol and $2 \times 10^7$ cells in a 20 $\mu$l volume were mixed with $8 \times 10^{-8}$ mol spin label. After 1 min of contact time, the cell suspension was transferred into a 50-$\mu$l micropipette capillary tube and sealed at the bottom with Critoseal (Syva Co., Palo Alto, CA). The micropipette with the cells was placed into the cavity of a Varian E-9 Century series spectrometer (Palo Alto, CA). ESR spectra were recorded at X-band, at 9.5 kHz, 100 field modulation, 4 G modulation amplitude, 100 G sweep range, and at 10 mW microwave power. The temperature of the probe was set to 24°C by the variable temperature accessory, using $N_2$ gas flow. Evaluation of the obtained ESR spectra, when 5-doxyl-SA was used, was by the equation expressing the order parameter, $S = 0.5407\ (T_{11} - T_1)/a_0$, where $a_0 = (T_{11} + 2T_1)/3$, and $T_{11}$ and $T_1$ are the outer and inner tensors obtained from the ESR spectra. When the T-SASL probe was used, the same instrument parameters were applied, except that the incident microwave power was 20 mW. The spectral parameters, $h_0$ and $h_{-1}$ represent spectral amplitudes and the ratio $h_0$:$h_{-1}$ defines the motional freedom of the probe according to Yin *et al.* (26). With both spin labels the ESR spectra show contributions from spin labels of restricted motion with no contribution from free-moving spin label.

**Lipid Packing of P-gp Expressing and Nonexpressing Cells.** Plasma membrane lipid packing can be studied by inserting the fluorescence probe merocyanine 540 (Molecular Probes) into cell membranes and assessing the degree of insertion as determined by fluorescence intensity using flow cytometry (27, 28). The experiment was according to Smith *et al.* (27) with some modifications. Briefly, $10^6$ cells suspended in 1 ml of PBS were treated with 10 $\mu$l of an MC540 stock solution at 1 mg/ml in 60% ethanol and 40% water. After a 10-min incubation at room temperature cells, were pelleted in a centrifuge, resuspended in 1 ml PBS, and fluorescence histograms were obtained. A Becton Dickson FacsCalibur flow cytometer was used at 488 nm excitation and 575 nm emission wavelength. Histograms were collected with $10^4$ cells. Results are expressed as means of fluorescence of the histograms.

**Determination of the Number of P-gp Molecules on Cell Membranes.** Determination of the number of P-gp molecules on the plasma membranes of cells was done according to the protocol of Bang Laboratories, Inc. Quantum 26 was as detailed by Weaver *et al.* (29). Standard fluorescent beads were obtained from Bang Laboratories (Fisher, IN). The supplied beads contained defined numbers of fluorescein molecules: 6318, 15877, 53589, 82914, 123338, 170473, 353992, and 437815. Fluorescence intensities of the beads were evaluated by flow cytometry using a FacsCalibur instrument. Beads were shaken vigorously before use and then mixed in pairs. Histograms were obtained by flow cytometry. Using the same instrument settings, the fluorescence intensity, means of histograms of cells labeled with MRK-16 mAb, and FITC-labeled antimouse $IgG_{2a}$ mAb (PharMingen, Becton Dickinson) were determined. Beads and the cells were suspended in the same PBS buffer for cytometry.

A standard curve was produced from the means of the histograms obtained with the beads, using semilogarithmic paper or by using QuickCal v2.1 data analysis software (Bangs Laboratories). Results of the two evaluations coincided. Mean fluorescence of the histograms obtained with the labeled cells was plotted on the standard curve, and numbers of P-gp molecules were read from the *Y*-axis. Consecutive determinations gave similar results.

## RESULTS

**Time-dependent Decrease of MDR1 mRNA after Tetracycline Treatment.** HeLa MDR-Off cells (see "Material and Methods") were treated with 2 $\mu$g/ml tetracycline to turn off expression of MDR1 mRNA in cells expressing high levels of both MDR1 mRNA and P-gp (see next section for protein data). Real-time quantitative PCR was performed on the samples using two different primer sets for MDR1 and one primer set for GAPDH as a control. The endogenous MDR1 primer set (MDR1–3′UTR) amplifies the region after the translation stop site that is not contained in the pRev-MDR1-Off plasmid. The total MDR1 primer set (MDR1) is within the protein-coding region and, therefore, amplifies MDR1 that was transfected as well as the endogenous MDR1. To demonstrate that the real-time PCR procedure is effective in calculating different amounts of target RNA, pRevTRE-MDR1 plasmid was used to make the copy number dilution series for the standard curve (Fig. 1*A*). Fig. 1*B* shows the data of the PCR done on the time course. Each point was normalized to its own GAPDH as a control for the RT-PCR reaction. The deviation of $\Delta$Cp at different times of tetracycline treatment from the $\Delta$Cp, where time of tetracycline treatment is equal to zero, was plotted against the time of tetracycline treatment to calculate the half-life of the message (Fig. 1*B*). The RNA half-life of this transcript was calculated to be 3.8 h. It should be pointed out that although these cells were selected with colchicine during their construction to select for the presence of the pRevTRE-MDR1 plasmid, the endogenous levels of MDR1 were not elevated by this selection.

**Measuring P-gp Expression and Function on the Cell Surface.** P-gp expression and transport function on the cell surface were assayed using FACS. For P-gp function, cells were incubated with rhodamine 123, a P-gp substrate, and accumulation in the cell was assayed. Cells that were grown in colchicine (high P-gp), or without colchicine but in tetracycline for 1–4 days (lower P-gp), and without colchicine or tetracycline for 4 days (high P-gp) were used (Fig. 2*A*). Percentage of rhodamine 123 accumulation is an inverse indication of

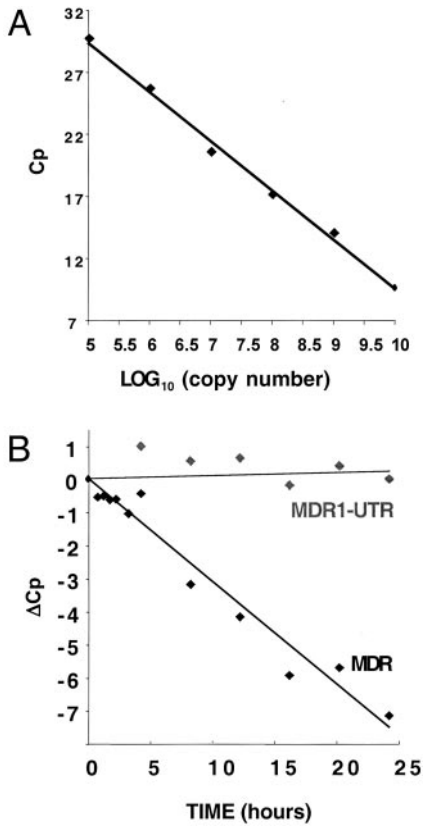


Fig. 1. Determination of the half-life of *MDR*1 mRNA in tet-repressible cells. *A*, demonstration of the linearity of the quantitative RT-PCR assay for the probes MDR1. $Cp = -3.9 \log_{10}$ (copy number) + 49.08. $R^2 = 0.9951$. *B*, effect of tetracycline on MDR1 transcript of HeLa P-gp$^{on}$ cells. MDR primers match both endogenous mRNA and plasmid mRNA. MDR-3′UTR primers match only endogenous MDR1 transcripts. The time course was performed after addition of 2 $\mu$g/ml tetracycline. Decay of MDR1 was calculated at the steady state, from 4 h to 24 h. $\Delta Cp_t - \Delta Cp_{t=0} = -0.3071.t + 0.1746$ $R^2 = 0.9116$.

P-gp function. Higher levels of rhodamine in the cells indicate decreased efflux of P-gp, or in this case decreased expression of P-gp. By the third day of tetracycline treatment, P-gp function was not detected, as indicated by the high accumulation of Rhodamine 123 in the cells. All of the cells were also treated with a P-gp inhibitor, cyclosporin A, to verify that that the assay was measuring P-gp function (data not shown).

P-gp expression was also measured with FACS using MRK16, a human-specific anti-P-gp mAb. The same cell conditions used in the rhodamine 123 accumulation assay were also used in the expression assay. Cells grown in colchicine and without drugs for 4 days expressed the most P-gp (Fig. 2*B*). Cells treated with tetracycline for 1–4 days show a steady decline of P-gp expression. Isotype controls were used, and the fluorescence after 4 days treatment with tetracycline was indistinguishable from cells not treated with any antibody or the internal fluorescence of the cells (data not shown). The decline of P-gp expression was plotted on a log scale, and the protein half-life was calculated to be 15.86 h. Similar results are observed in the mouse 77.1 Tet-Off cell line (data not shown). Western blots were used to confirm the results observed in the FACS assays (Fig. 2*C*). HeLa P-gp$^{on}$ cells were treated with 2 $\mu$g/ml tetracycline for 1 day, 2 days, 3 days, and 4 days. GAPDH antibody was used as a protein loading control. On the basis of these results, for the rest of the experiments day 0 cells were not incubated with tetracycline and are denoted as HeLa P-gp$^{on}$ and 77.1 P-gp$^{on}$. Day 4 cells were incubated with tetracycline for 4 days and are denoted as HeLa P-gp$^{off}$ and 77.1 P-gp$^{off}$.

**HeLa P-gp$^{on}$ Cells Are Multidrug Resistant.** HeLa MDR-Off cells were either grown in 20 ng/ml colchicine (HeLa P-gp$^{col}$), 2 $\mu$g/ml tetracycline (HeLa P-gp$^{off}$), or no drug (HeLa P-gp$^{on}$) for at least 4 days before starting the cloning assay. The cells grown in colchicine are expressing as much P-gp as the ones grown out of drug; the nomenclature is used to specify what medium was used. Colony-forming assays with the cell lines HeLa P-gp$^{col}$, HeLa P-gp$^{on}$ and HeLa P-gp$^{off}$, were done, individually, with increasing concentrations of three known MDR substrates: colchicine, doxorubicin, and vinblastine (Fig. 3, *A–C*). The percentage of relative cloning efficiency was calculated and plotted against the concentration of colchicine used on a log scale (Fig. 3*A*). After the cells were treated for 4 days under different conditions, tetracycline was either present or absent (−t) from the medium for the 8–10 days that the colonies were

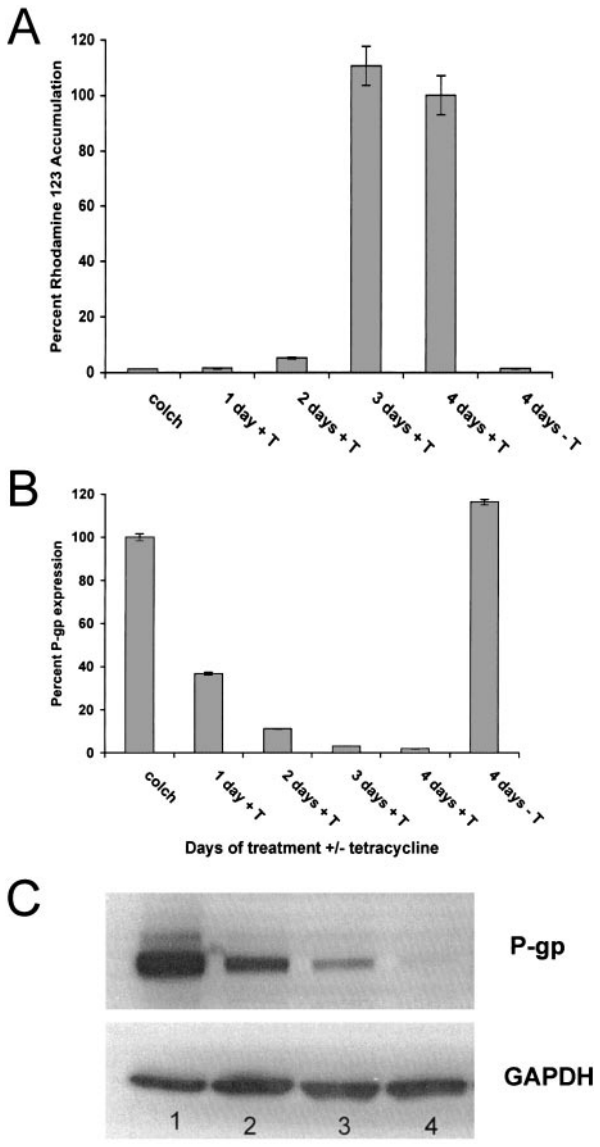


Fig. 2. Determination of time needed to return P-gp expression on tet-repressible cells to basal levels. *A*, to assay P-gp function, the ability of P-gp to pump out rhodamine 123 was studied. FACS was used to measure accumulation of rhodamine 123. HeLa P-gp$^{on}$ cells were treated up to 4 days in 2 $\mu$g/ml tetracycline (+T). Controls used were cells grown in colchicine (*colch*) and cells grown in the absence of drugs (−T). *B*, P-gp expression was also measured in a FACS assay with MRK16 (a mAb against human P-gp). *C*, a Western blot was used to verify the decrease of P-gp expression observed in the FACS assay. C219, another mAb against P-gp, was used to detect P-gp on blots. As a control for loading, an antibody against GAPDH was used. *Lanes 1–4* are cells treated with 2 $\mu$g/ml tetracycline for 1, 2, 3, and 4 days, respectively; *bars*, ±SD.

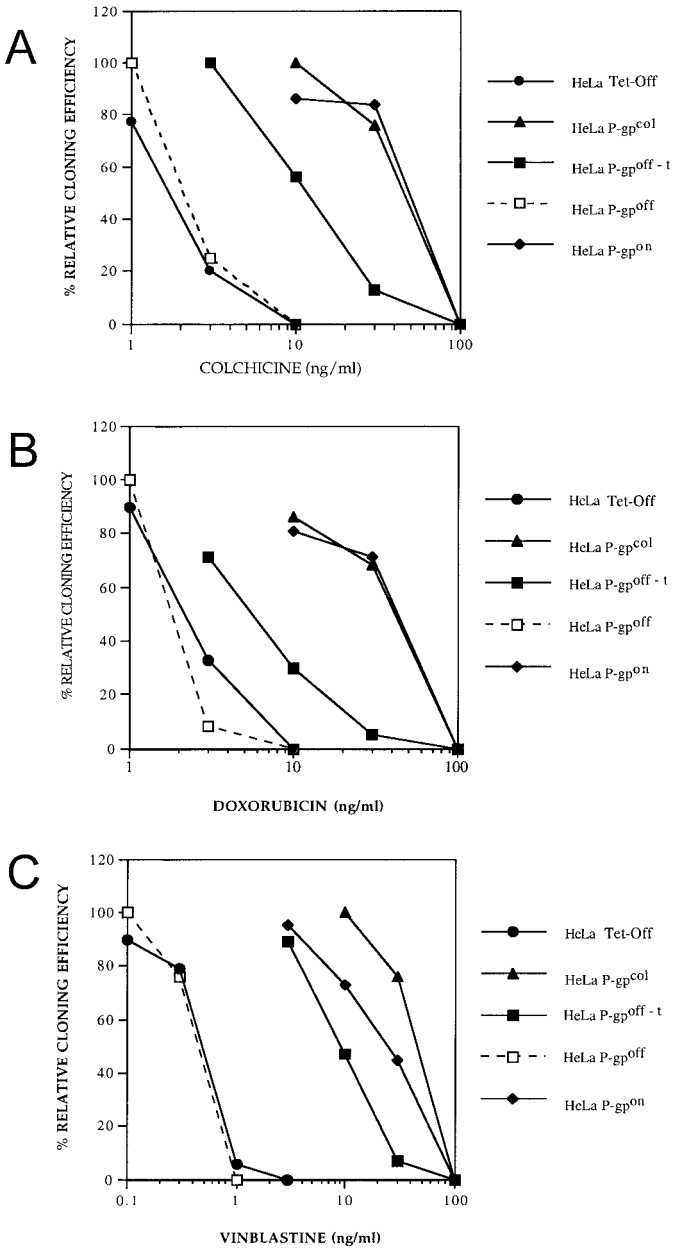


Fig. 3. Tet-repressible cells are MDR as determined by colony forming assays. HeLa Tet-Off, the parental cells, (●) and HeLa MDR-Off cells with different treatments were assayed for resistance to increasing amounts of (*A*) colchicine, (*B*) doxorubicin, and (*C*) vinblastine. HeLa P-gp$^{col}$ (▲) were cultured in colchicine, HeLa P-gp$^{off}$ (□) were cultured in 2 $\mu$g/ml tetracycline for the 8–10-day cloning assay, and HeLa P-gp$^{on}$ (♦) were cultured in the absence of all drugs 4 days before the cloning assay. HeLa P-gp$^{off\text{-}t}$ (■) cells were cultured in 2 $\mu$g/ml tetracycline before the cloning assay; for the duration of the cloning assay, tetracycline was removed; *bars*, ±SD.

established. These series of figures show that the resistance of the cell lines is controlled by the presence or absence of tetracycline. When P-gp is turned off (HeLa P-gp$^{off}$) before the cloning assay, it stays off in the presence of tetracycline, and the level of resistance is similar to that of the parental HeLa Tet-Off cells in all three of the drug conditions used (Fig. 3, *A–C*). This demonstrates that the period of colchicine selection of this cell line did not affect the expression of endogenous MDR1. It is interesting to note that in all three of the treatments, when HeLa P-gp$^{off}$ are taken out of tetracycline for the remainder of the cloning assay (8–10 days) resistance starts to come back, indicating that P-gp expression is returning and that this system is very well regulated by tetracycline treatment (HeLa P-gp$^{off\text{-}t}$ in Fig. 3, *A–C*).

**Quantitation of P-gp Molecules on P-gp$^{on}$ Cells, P-gp$^{off}$ Cells, and Cells Selected in Cytotoxic Drugs.** The number of P-gp molecules was estimated from fluorescence intensities of FITC-mAb-labeled cells by comparing to a standard curve, obtained from beads with a predetermined number of FITC molecules. These estimates indicate that the level of P-gp cell surface expression in the HeLa P-gp$^{on}$ cells in the absence of tetracycline ($2 \times 10^5$ P-gp molecules/cell) is comparable with the level of cell surface P-gp in KB-C1 cells ($4.1 \times 10^5$ P-gp molecules/cell). In the P-gp$^{off}$ cells the amount of P-gp in the surface ($<5 \times 10^3$ P-gp molecules/cell) is comparable with the KB-3–1 cells ($6 \times 10^3$ P-gp molecules/cell) that are not MDR. We conclude that the level of P-gp expression in HeLa P-gp$^{on}$ is similar to those of long-term drug-selected cells.

**Membrane Potential of Drug Selected and Tet-repressible Cells.** A representative set of results of relative membrane potentials of P-gp nonexpressing cells (KB-3–1, KB-A1rev, 77.1 P-gp$^{off}$, and HeLa P-gp$^{off}$) and P-gp expressing cells (KB-V1, KB-A1, HeLa P-gp$^{on}$, and 77.1 P-gp$^{on}$) using the fluorescent membrane potential probe, oxonol, are shown in Fig. 4. Similar results were obtained with cells grown in separate cultures ($n$ = 3–5). There is a reproducible difference in membrane potentials between drug-selected, P-gp expressing cells, KB-V1 and KB-A1 (Fig. 4, *Lanes 2* and *4*), and the P-gp nonexpressing cells, KB-3–1 and KB-A1rev (Fig. 4, *Lanes 1* and *3*). Lower fluorescence of the drug-selected cells indicates a relative hyperpolarization as compared with their parental cells and revertant control cells. Contrary to the results with the drug-selected cells, 77.1 P-gp$^{on}$ and HeLa P-gp$^{on}$ cells (Fig. 4, *Lanes 6* and *8*) have similar fluorescence intensity to 77.1 P-gp$^{off}$ and HeLa P-gp$^{off}$ (Fig. 4, *Lanes 5* and *7*), suggesting that the membrane potential is similar in both conditions regardless of P-gp presence or absence.

The oxonol dye is not a substrate of P-gp. This fact is supported by the fact that both sets of tet-repressible cells do not differ in their fluorescence intensity, as shown in Fig. 4, *Lanes 5–8*. Also, ATPase stimulation studies with partially purified membrane-containing P-gp indicated little or no stimulation with 500 nM oxonol compared with the 150 nM oxonol used to measure membrane potential (data not shown).

**Motional Freedom of ESR Probes in the Plasma Membranes of P-gp Expressing and Nonexpressing Drug-selected and -transfected Cells.** The motional freedom of the ESR probe, 5-doxyl-SA, expressed as the order parameter, S, indicates the fluidity of the plasma membranes of the cells. Fig. 5 shows the calculated S values of the studied cells. Drug-selected KB-V1 (Fig. 5, *Lane 4*) cells have lower S values indicating more fluid plasma membranes. The KB-A1

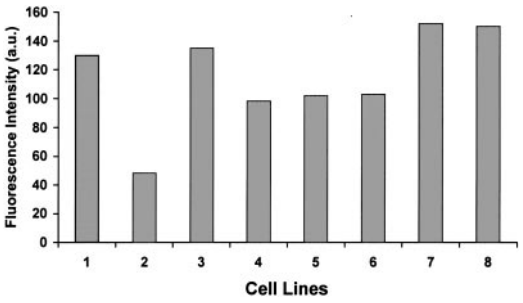


Fig. 4. Membrane potential measured with oxonol. Membrane potential of P-gp-expressing, drug-selected cells, KB-V1 (*Lane 2*) and KB-A1 (*Lane 4*), tet-repressible cells, 77.1 P-gp$^{on}$ (*Lane 6*) and HeLa P-gp$^{on}$ (*Lane 8*), and the control cells not expressing P-gp, KB-3–1 (*Lane 1*), KB-A1rev (*Lane 3*), 77.1 P-gp$^{off}$ (*Lane 5*), and HeLa P-gp$^{off}$ (*Lane 7*). Membrane potential was measured by flow cytometry, using oxonol (150 nM) as a membrane potential sensing dye, as detailed in "Materials and Methods." Results show typical fluorescence intensities of a series of measurements with the cells ($5 \times 10^5$ cells/ml). Several measurements with cells grown separately gave similar fluorescence intensity relations and relative membrane potentials ($n$ = 3).

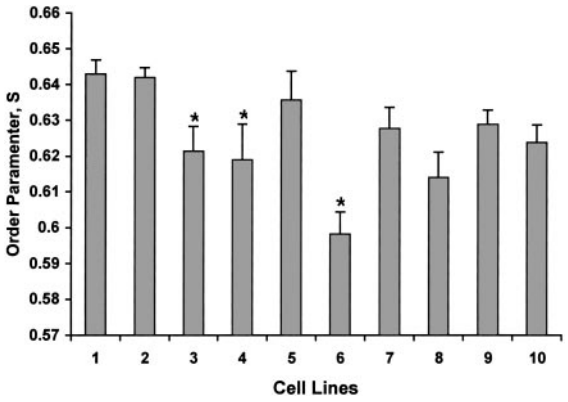


Fig. 5. Determination of motional freedom of ESR probe 5-doxyl-SA in tet-repressible and drug-selected cells. Motional freedom, as expressed by the order parameter, *S*, determined with the ESR probe 5-doxyl-SA inserted into plasma membranes of cells. Experimental details, and calculation of the order parameter are described in "Materials and Methods." KB-3-1 cells (*Lane 1*), KB-A1rev (*Lane 5*), HeLa P-gp$^{off}$ (*Lane 7*), and 77.1 P-gp$^{off}$ (*Lane 9*) are P-gp nonexpressing cells, whereas KB-8–5 (*Lane 2*), KB-C1 (*Lane 3*), KB-V1 (*Lane 4*), and KB-A1 (*Lane 6*) are drug-selected cells that express P-gp in increasing amounts. Tet-repressible cells expressing P-gp are HeLa P-gp$^{on}$ (*Lane 8*) and 77.1 P-gp$^{on}$ (*Lane 10*). Mean order parameters were calculated from results obtained with separately grown cell-cultures ($n = 3–4$). *t* tests indicate $P < 0.05$ (∗) for the KB-8–5 and KB-C1 pair (∗); KB-3-1 and KB-V1 (∗); KB-A1 and KB-A1rev (∗). There were no significant differences between HeLa and 77.1 P-gp$^{off}$ *versus* P-gp$^{on}$; *bars*, ±SD.

drug-selected cells (Fig. 5, *Lane 6*) also have more fluid plasma membranes than the KB-A1revertant (Fig. 5, *Lane 5*). SDs indicate statistical significance ($P < 0.05$; $n = 3–5$). Contrary to these results, the difference in the S values between the HeLa P-gp$^{on}$ (Fig. 5, *Lane 8*) compared with HeLa P-gp$^{off}$ (Fig. 5, *Lane 7*), and between 77.1 P-gp$^{on}$ (Fig. 5, *Lane 10*) and 77.1 P-gp$^{off}$ (Fig. 5, *Lane 9*) showed small differences that failed to reach statistical significance ($P = 0.06$ and 0.075, respectively).

To confirm the results obtained with the 5-doxyl-SA probes that drug-selected cells have more fluid plasma membranes, another ESR probe, which probes at the outer surface of the plasma membrane, T-SASL, was also used. The motional freedom of the T-SASL probe is expressed as the spectral parameters, $h_0/h_{-1}$ according to Yin *et al.* (26). The data indicate that the KB-3-1 ($h_0:h_{-1} = 2.47 \pm 0.1$) and low-level resistance KB-8-5 ($h_0:h_{-1} = 2.45 \pm 0.08$) cells are more rigid with higher $h_0:h_{-1}$ ratio numbers, than highly drug-selected KB-C1 ($h_0:h_{-1} = 2.26 \pm 0.06$) and KB-V1 ($h_0:h_{-1} = 2.24 \pm 0.05$) cells ($P < 0.05$ between drug sensitive, KB-3-1, and drug resistant, KB-C1). These results parallel those obtained with the 5-doxyl-SA probe and support the conclusions that drug-selected cells have more fluid membranes than the nonexpressing P-gp cells.

**Motional Freedom of Fluorescent Molecules as Determined by Polarization Experiments.** The TMA-DPH fluorescent probe was used as an alternative way to measure the fluidity of plasma membranes, because this probe is known to probe only at the surface of the plasma membrane and does not translocate deeper into membranes (24). Fig. 6*A* shows the results of fluorescence polarization obtained with drug-selected and tet-repressible *MDR*1 gene-transfected cells. Whereas there is a significant difference between parental, KB-3-1 and KB-A1rev (Fig. 6*A*, *Lanes 1* and *3*) and the drug selected, P-gp-expressing KB-V1 and KB-A1 cells (Fig. 6*A*, *Lanes 2* and *4*), no significant difference can be detected between the tet-repressible HeLa P-gp$^{on}$ and 77.1 P-gp$^{on}$ (Fig. 6*A*, *Lanes 6* and *8*) compared with HeLa P-gp$^{off}$ and 77.1 P-gp$^{off}$ cells (Fig. 6*A*, *Lanes 5* and *7*). These results, with a cell surface probe, suggest decreased fluidity of drug-selected cells, in contrast with previous results with ESR probes, which indicated increased fluidity.

Results of polarization experiments using another fluorescence anisotropy probe, (AOSA), are shown in Fig. 6*B*. This probe was used to clarify apparent differences between the ESR results (Fig. 5) and the polarization results obtained with the TMA-DPH probe (Fig. 6*A*). Polarization numbers are lower for drug-selected P-gp-expressing KB-C1 and KB-V1 cells (Fig. 6*B*, *Lanes 3* and *4*) than for P-gp nonexpressing KB-3-1 and KB-A1rev cells (Fig. 6*B*, *Lanes 1* and *5*). These results parallel those obtained with both the 5-doxyl-SA (Fig. 5) and T-SASL ESR probes. However, these results are different from the results obtained with the TMA-DPH polarization probe, which probes only the membrane surface (Fig. 6*A*). It appears, then, that motional freedom of both the polarization and ESR probes indicate similar increase in membrane fluidity inside the plasma membranes of drug-selected cells.

**Membrane Lipid Packing of P-gp Expressing and Nonexpressing Cells.** Lipid packing of parental and drug-selected cells expressing different amounts of P-gp was studied to determine whether the different cell lines expressing different amounts of P-gp have different biophysical status of their plasma membranes as shown by ESR and polarization, and also insert different amounts of the lipid packing probe, merocyanin 540. Fig. 7 shows typical results of means of

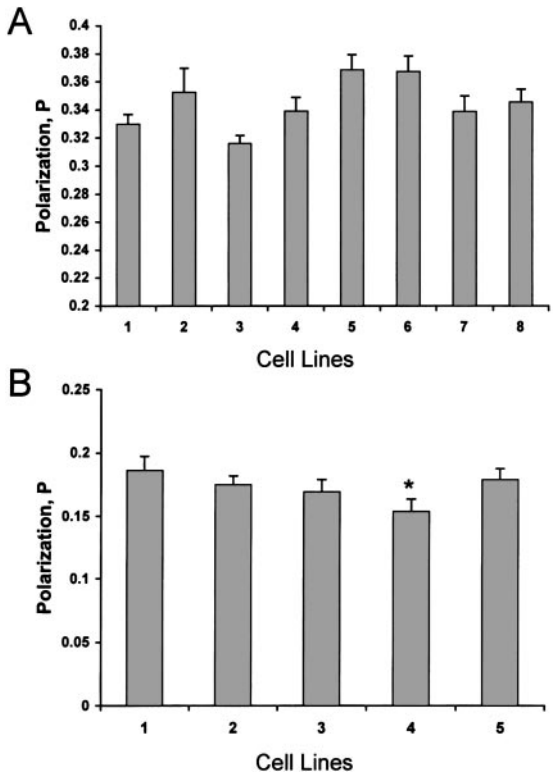


Fig. 6. Polarization of TMA-DPH and AOSA probes in cells. *A*, polarization of the TMA-DPH fluorescence probe at the surface of the plasma membrane of cells. KB-3-1 (*Lane 1*), KB-A1rev (*Lane 3*), HeLa P-gp$^{off}$ (*Lane 5*), and 77.1 P-gp$^{off}$ (*Lane 7*) cells are P-gp-nonexpressing cells. KB-V1 (*Lane 2*) and KB-A1 (*Lane 4*) are drug-selected, and HeLa P-gp$^{on}$ (*Lane 6*) and 77.1 P-gp$^{on}$ (*Lane 8*) are the tet-repressible P-gp-expressing cells. Mean polarizations were calculated from results obtained with separately grown cells ($n = 2$) and several measurements with cells from the same culture ($n = 4–6$). Statistical analysis indicates significant differences, $P < 0.05$ between KB-3-1 and KB-V-1 cells, and between KB-A1rev and KB-A1 cells. However, there is no significant difference between 77.1 P-gp$^{on}$ and 77.1 P-gp$^{off}$ cells, and between HeLa P-gp$^{on}$ and HeLa P-gp$^{off}$ cells. *B*, polarization of the plasma membranes was determined by using AOSA probe that inserts in the membranes. KB-3-1 and KB-A1rev cells (*Lanes 1* and *5*) are P-gp nonexpressing, and KB-8-5, KB-C1, and KB-V1 (*Lanes 2*, *3*, and *4*, respectively) are P-gp-expressing cells. Mean polarization numbers were obtained from two independently grown cell cultures, and several measurements were made with each cultured cell population ($n = 3–4$). Statistical evaluation indicates that there is significant difference, $P < 0.05$, between KB-3-1 or KB-A1rev and KB-V-1 cells. Statistical differences between other cells are not significant at the level of $P < 0.05$. Experimental details and calculation of polarization, *P*, for both *A* and *B* are described in "Materials and Methods;" *bars*, ±SD.

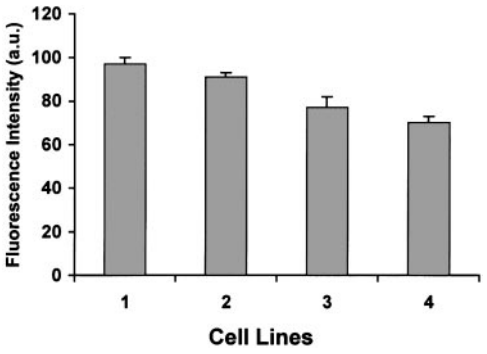


Fig. 7. Membrane lipid packing as expressed by fluorescence intensity of Merocyanin 540 stained cells. Experimental details are given in "Materials and Methods." KB-3-1 (*Lane 1*) is a P-gp-nonexpressing parental cell, and KB-8-5, KB-C1, and KB-V1 (*Lanes 2*, *3*, and *4*, respectively) are P-gp-expressing drug-selected cells. One typical result of several, obtained with separately grown cells, is shown ($n = 3$); *bars*, ±SD.

fluorescent histograms collected from $10^4$ cells from each cell line ($n = 3–4$). Results indicate that the drug-selected P-gp-expressing cells (Fig. 7, *Lanes 2–4*) incorporate less merocyanine 540 than the P-gp nonexpressing KB-3-1 cells (Fig. 7, *Lane 1*), indicating that non-P-gp-expressing cells have loosely packed lipids compared with P-gp-expressing cells.

## DISCUSSION

These studies confirm that there are differences in the biophysical properties of plasma membranes of drug-sensitive and MDR cells including increased membrane potential and measured membrane fluidity of the drug-selected cells. Because all seven of the drug-resistant cell lines studied express P-gp, the multidrug efflux pump, we used a tet-repressible plasmid carrying *MDR*1 to determine whether P-gp expression was responsible for these differences in biophysical parameters. The results indicate that HeLa P-gp$^{on}$, which has P-gp levels comparable with a HeLa subclone, KB-C1, do not show these differences compared with the same cells in which P-gp expression has been turned off by tetracycline treatment in HeLa P-gp$^{off}$. These results help to clarify the physiological role of P-gp and indicate that cells selected to high levels of resistance for long periods of time in cytotoxic drugs, such as vinblastine, doxorubicin, and colchicine, likely have differences in membrane properties, which are not attributable simply to P-gp expression.

To compare P-gp expressing and nonexpressing cells in the same genetic background, we used a tet-repressible plasmid system and achieved high-level expression of P-gp in the absence of tetracycline, which could be repressed after 4 days of tetracycline treatment. The data clearly demonstrate that the P-gp expression and MDR phenotype demonstrated in the tet-repressible cells was because of transcription from the transfected plasmid and that endogenous *MDR*1 mRNA levels were not affected by the tetracycline treatment or colchicine selection. It is also very clear that the total *MDR*1 mRNA decreases exponentially with time in tetracycline treatment. *MDR*1 mRNA began to decline logarithmically within a few hours of tetracycline treatment with a half-life of 3.8 h. Previous studies have reported RNA half-life for *MDR*1 mRNA to range between 1 and 8 h (30, 31), but in this case we cannot compare our data with the half-life of the endogenous *MDR*1 mRNA, because the *MDR*1 mRNA encoded by the tet-repressible vector system is not identical in its noncoding regions to the endogenous *MDR*1 mRNA. However, these mRNA half-life data indicate that transcription is turned off completely by tetracycline treatment, and that loss of P-gp from the cell surface and its degradation becomes the limiting determinant of P-gp function. On the basis of our analysis of cell-surface P-gp, we found a surface half-life of 15.8 h. These data concur with the estimate of 17.5 h in CH$^R$C5 cells and 18.2 h in CEMVLBO.1 cells, which are both drug-selected cell lines (32). Others report a P-gp protein half-life that range between 24 h and 72 h on different cell lines and under different conditions (31, 33, 34). The longer half-lives in some drug-selected cells suggest the possibility that selection favors host cell alterations that extend the half-life of P-gp. There is another study that estimated the P-gp half-life to be 3 h using a pulse chase method in a vaccinia virus expression system that may be a result of the transient transfection system (35).

Fig. 2, *A* and *B* clearly demonstrate that as P-gp expression decreases, rhodamine 123 accumulation increases. To avoid long-term drug selection effects of colchicine, which was used as a selection to introduce the tet-repressible plasmid into cells, we kept track of cell passages and only used cells grown up to 28 passages in colchicine. Because HeLa P-gp$^{off}$ cells are as sensitive to colchicine as the parental HeLa Tet-Off cells (Fig. 3*A*), the effects of the initial selection on expression must be minimal.

Membrane fluidity was studied by determining the motional freedom of ESR probes, 5-doxyl SA (Fig. 5) and T-SASL, in a series of cells expressing increasing amounts of P-gp on their cell surface and compared with results obtained with the Tet-repressible cells. We have shown that whereas the membrane is increasingly more fluid as drug-selected cells become more resistant (and express more P-gp), the fluidity changes between HeLa P-gp$^{on}$ and P-gp$^{off}$, and mouse 77.1 P-gp$^{on}$ and P-gp$^{off}$ cells are not statistically significant. Interestingly, ESR studies also using the 5-doxyl-SA probe were used by Siegfried *et al.* (36) to show correlation between doxorubicin sensitivity and plasma membrane fluidity.

Results similar to those from the ESR probe were obtained when fluorescence polarization of membrane-inserted molecules were studied using 6-(9-anthroyloxy) SA (Fig. 6*B*). Again, cells of increasing drug resistance gave increasingly lower polarization numbers, indicating more fluid plasma membranes, in agreement with the ESR results. In contrast, the polarization probe TMA-DPH yielded opposite results when we compared the fluidity of KB-3-1 and KB-V1 cells (Fig. 6*A*). However, whereas 6-(9-anthryloxy) SA probes are six carbons deep in the plasma membrane, TMA-DPH probes only at the surface of the plasma membrane (24). We speculate that TMA-DPH may bind cell surface proteins that are expressed at higher levels in the drug-selected cells, and this binding restricts its motion, yielding higher polarization numbers, interpreted as higher rigidity of plasma membranes. No significant difference was found between polarization of TMA-DPH in the HeLa and 77.1 P-gp$^{on}$ and P-gp$^{off}$ cells with this probe (Fig. 6*A*). This result indicates that TMA-DPH is not a substrate of P-gp and that P-gp may not be the putative surface protein or proteins to which TMA-DPH molecule binds. Lipid packing of merocyanin 540 also indicates differences among drug-selected MDR cells. This result is consistent with the established differences in the plasma membrane found by the polarization and the ESR experiments discussed above.

Membrane potential measurements also indicate that expression of P-gp alone does not alter the membrane potential of the plasma membranes, and perhaps other changes in membrane composition are responsible for these differences. Fig. 4 shows that whereas there is a substantial difference between drug-selected P-gp-expressing cells, KB-V1 and KB-A1, and nonexpressing cells, KB-3-1 and KB-A1rev, in their uptake of the membrane potential sensing dye oxonol, no such difference exists between the pairs of tet-repressible cells. The drug-selected, P-gp-expressing cells, KB-V1 and KB-A1 cells, have higher membrane potential, indicating that the cytoplasm is more negatively charged. This observation has been associated with the proton extru-

sion ability of P-gp expressing cells, as shown by the experiments of Landwojtowicz *et al.* (37). However, since there is no significant membrane potential difference in the human and mouse P-gp tet-repressible cells, one can argue that P-gp is not the proton pump responsible for the membrane potential differences observed in drug-selected cells (Fig. 4).

What changes in drug-selected cells might be responsible for the changes in membrane fluidity and membrane potential? Most likely, there are other cellular alterations that help confer drug resistance in the presence of P-gp. These could be changes in other membrane proteins, membrane-anchoring proteins (19), or in the composition of membrane lipids themselves (19). However, because the cell lines KB-C1 and KB-V1 can have their resistance completely reversed by inhibition of P-gp, it seems likely that these putative membrane changes that affect fluidity and potential do not confer resistance by themselves, but work to enhance the effect of P-gp. For example, increased membrane fluidity would enhance delivery of xenobiotics to P-gp for excretion; increased membrane potential would help keep negatively charged organic cations out of cells after they are pumped. It is possible that alterations in the plasma membrane biophysical properties may help the functionality of P-gp. Callaghan *et al.* (38) showed that incorporation of fatty lipids into the plasma membrane, which made the membranes less fluid, suppressed the function of the expressed P-gp molecules. Finally, it is also possible that sustained expression of P-gp at the cell surface has a deleterious effect on cell growth, and the changes in biophysical properties of the membrane may help ameliorate these negative effects of P-gp. The tet-repressible expression system would not uncover such evolutionary adaptations seen in cells subject to drug selection over long periods of time.

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