# P-Glycoprotein-Mediated Colchicine Resistance in Different Cell Lines Correlates with the Effects of Colchicine on P-Glycoprotein Conformation†

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*Received June 15, 2000; Revised Manuscript Received November 7, 2000*

ABSTRACT: The multidrug transporter P-glycoprotein (Pgp) is an ATPase efflux pump for multiple cytotoxic agents, including vinblastine and colchicine. We have found that resistance to vinblastine but not to colchicine in cell lines derived from different types of tissues and expressing the wild-type human Pgp correlates with the Pgp density. Vinblastine induces a conformational change in Pgp, evidenced by increased reactivity with a conformation-sensitive monoclonal antibody UIC2, in all the tested cell lines. In contrast, colchicine increases the UIC2 reactivity in only some of the cell lines. In those lines where colchicine alone did not affect UIC2 reactivity, this drug was, however, able to reverse the vinblastine-induced increase in UIC2 reactivity. The magnitude of the increase in UIC2 reactivity in the presence of saturating concentrations of colchicine correlates with the relative ability of Pgp to confer colchicine resistance in different cell lines, suggesting the existence of some cell-specific factors that have a coordinate effect on the ability of colchicine to induce conformational transitions and to be transported by Pgp. Colchicine, like vinblastine, reverses the decrease in UIC2 reactivity produced by nonhydrolyzable nucleotides, but unlike vinblastine, it does not reverse the effect of ATP at a high concentration. Colchicine, however, decreases the Hill number for the effect of ATP on the UIC2 reactivity from 2 to 1. Colchicine increases the UIC2 reactivity and reverses the effect of ATP in ATPase-deficient Pgp mutants, but not in the wild-type Pgp expressed in the same cellular background, suggesting that ATP hydrolysis counteracts the effects of colchicine on the Pgp conformation.

The multidrug transporter Pgp[1] is an ATPase efflux pump for multiple cytotoxic agents, responsible for the best-known form of multidrug resistance in tumor cells (*1*, *2*). Pgp is a 170 kDa glycoprotein consisting of two homologous halves, each containing a nucleotide-binding domain with NBS-carrying consensus Walker A and Walker B sequence motifs, characteristic of the ATP-binding cassette (ABC) family of transport proteins (*3*), and a hydrophobic domain with six transmembrane segments. It has been demonstrated through chemical means (*4*) or by amino acid substitutions located in either the N-terminal or C-terminal Walker A motif (*5*, *6*) that both NBS must be intact for Pgp to hydrolyze ATP. The presence of Pgp transport substrates has been shown to increase the rate of ATP hydrolysis by Pgp (*5*, *7*). A mutation that alters the relative ability of Pgp to confer resistance to different drugs was also found to change the ability of such drugs to stimulate ATP hydrolysis (*5*). While many mutations that affect drug transport by Pgp have been identified (*8*), it is not known whether external factors may also affect the relative efficacy of drug transport by Pgp. Romsicki and Sharom (*9*) have shown that the relative binding affinity of different drugs for purified Pgp in lipid mixtures was affected by the lipid composition, suggesting that Pgp−drug interactions may also vary among cell types with different membrane lipid compositions.

We have previously shown that the reactivity of Pgp encoded by the human MDR1 gene with a conformation-specific monoclonal antibody UIC2 is affected by different Pgp ligands (*10*, *11*). Specifically, the UIC2 reactivity is decreased by different Pgp-binding nucleotides, whereas Pgp substrates, such as vinblastine, reverse this effect of nucleotides and increase the UIC2 reactivity. While most of the Pgp-transported drugs were found to increase the UIC2 reactivity in intact Pgp-expressing cells, three of these substrates (colchicine, etoposide, and puromycin) failed to increase the UIC2 reactivity in the original survey (*10*). In the study presented here, we have found that colchicine can increase the UIC2 reactivity in some but not all Pgp-expressing cell lines, and that the ability of colchicine to increase the UIC2 reactivity correlates with its relative ability to be transported by Pgp in different cells. We have also used the UIC2 reactivity shift assay to characterize the effects of colchicine in the presence of vinblastine, ATP, or nonhydrolyzable nucleotides. The results of these assays

† This work was supported by NIH Grant R37CA40333 (I.B.R.). W.D.S. was the recipient of a Yamagiwa-Yoshida Memorial International Cancer Study grant administered by the International Union Against Cancer (UICC).

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[1] Abbreviations: Pgp, P-glycoprotein; FACS, fluorescence-activated cell sorter; ABC, ATP-binding cassette; NBS, nucleotide-binding site(s); AMP-PNP, 5′-adenylylimidodiphosphate; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid.

10.1021/bi001372n CCC: $20.00 
Published on Web 03/13/2001

indicate that ATP hydrolysis by Pgp counteracts the effects of colchicine on the Pgp conformation.

## EXPERIMENTAL PROCEDURES

*Materials.* ATP, AMP-PNP, vinblastine, colchicine, and propidium iodide were from Sigma. *Staphylococcus aureus* α-toxin was purchased from List Biological Labs. The goat anti-mouse IgG2a fluorescein isothiocyanate (FITC)-conjugated secondary antibody was obtained from Caltag Laboratories. MRK16 monoclonal antibody was generously provided by T. Tsuruo (University of Tokyo, Tokyo, Japan). The isolation and preparation of the monoclonal antibody UIC2 have been previously described (*12*).

*Cell Lines.* LMtk$^-$ murine fibroblast cell lines, KK-H, KK-L, KM-H, MK-H, and MM, were derived after transfection with either the wild-type (KK) or mutant (KM, MK, and MM) forms of human MDR1 cDNA, followed by vinblastine selection or (in the case of MM) by FACS sorting for the expression of human Pgp (ref *10* and unpublished data). The MK, KM, and MM mutants contain amino acid substitutions at either one (KM or MK) or both (MM) conserved lysine residues in the Walker A motifs of the N-terminal or C-terminal NBS, K433M and K1076M, respectively.

HT1080-MDR1 cells constitute a population of HT1080 human fibrosarcoma cells infected with a recombinant retroviral vector carrying human *MDR*1 cDNA, with Pgp-positive cells isolated with the FACS (*13*). 3T3-MDR1 cells were derived without cytotoxic selection from mouse NIH 3T3 fibroblasts as described in ref *14*. The KB-GRC1 cell line was derived following transfection of KB-3-1 cells with human *MDR*1 cDNA and a single step of colchicine selection (*15*).

The K562/i-S9 cell line was derived from human K562 leukemia cells by infection with a recombinant retrovirus carrying the human MDR1 cDNA followed by subcloning (without cytotoxic selection) and immunostaining for Pgp (*16*). The multidrug-resistant CEM/VLB-100 cell line was derived from human T-lymphoblastoid CEM leukemia cells by multistep selection with vinblastine (*17*).

All attached cell lines were maintained in 15 cm tissue culture plates in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% glutamine, and a 1% penicillin/streptomycin mixture. Leukemic cells that grow in suspension were maintained in 75 cm$^2$ flasks in RPMI 1640 medium containing 10% fetal bovine serum, 1% glutamate, and a 1% penicillin/streptomycin mixture.

*Fluorescence-Activated Cell Sorting Assays*. All primary antibody staining reactions were carried out in a final volume of 200 μL containing $1 \times 10^6$ cells/reaction mixture in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). Cells were reacted with their respective nucleotides and/or drugs for 10−15 min at 37 °C prior to the addition of the primary antibody. UIC2 was aliquoted, heated at 48 °C for 24 h in a thermal cycler, and stored at 4 °C prior to use. This treatment did not affect the reactivity of the antibody to Pgp in the presence of substrate, but decreased the reactivity of the antibody to Pgp in the absence of substrate, thus increasing the sensitivity of the assay (our unpublished data). The primary antibody concentration that was used (15 μg/mL) had been previously determined to be a saturating antibody concentration for all available binding sites on $1 \times 10^6$ cells. Following addition of the primary antibody, the reaction mixture was held at 37 °C for an additional 30 min. Primary antibody reactions were stopped by the addition of 5 mL of ice-cold PBS and the cells then centrifuged for 5 min at 4 °C and 1500 rpm. Pellets were resuspended in 100 μL of PBS and 1% BSA buffer containing 25 μg/mL goat anti-mouse FITC-conjugated secondary antibody. The reaction mixtures were left on ice, to prevent the function of Pgp, for 30 min before the reactions were stopped by the addition of 5 mL of ice-cold PBS buffer and the mixtures centrifuged for 5 min at 4 °C and 1500 rpm. Immediately prior to FACS analysis, each pellet was resuspended in 350−500 μL of ice-cold PBS and 1% BSA buffer containing 1 μg/mL propidium iodide (PI) and left on ice. Two-color cytofluorometric analysis was performed by acquiring at least 10 000 individual events using a Becton Dickinson FacSort flow cytometer. Flow cytometric data were analyzed by using the Becton Dickinson Information Systems CellQuest software.

*Data Analysis.* We determined the kinetic parameters from the data using the SigmaPlot program. This gave us (1) the maximum or minimum fluorescence ($F_{max}$ or $F_{min}$, respectively) at asymptotically high or low levels of vinblastine, nucleotide, or vanadate, (2) the concentration of vinblastine, nucleotide, or vanadate at which half of the maximal change in fluorescence occurs ($K_m$), and (3) the Hill number, $n$. The best-fit regression through the respective data points was determined using the appropriate binding isotherm, as follows. We used eq 1 below for cases where the fluorescence signal, $F$, increases with $B$, the concentration of ligand, and eq 2 for those cases in which the fluorescence decreases, as stated in the text.

$$F = F_{min} + [(F_{max} - F_{min})B^n]/(K_m{}^n + B^n) \quad (1)$$

$$F = F_{min} + [(F_{max} - F_{min})K_m{}^n]/(K_m{}^n + B^n) \quad (2)$$

*S. aureus* α*-Toxin Permeabilization.* The concentration and time course of *S. aureus* α-toxin necessary to yield an approximate 50% distribution between PI-positive and PI-negative staining cells was determined for each cell line in preliminary experiments. An α-toxin concentration was chosen that was effective in 15−30 min at 37 °C. The approximate percentage of permeabilization was checked periodically under a light microscope via trypan blue staining and counting the percentage of blue cells per high power field. Permeabilization was performed in PBS and 1% BSA buffer at a final volume of 100 μL containing $1 \times 10^6$ cells at 37 °C. The reaction was stopped by the addition of 30 mL of 37 °C PBS, and the cells were centrifuged for 5 min at 1500 rpm and room temperature. Pellets were resuspended in PBS containing 10 mM $MgCl_2$ with or without the indicated nucleotide at 37 °C for 15 min, and then each reaction proceeded through the FACS assay procedure as described above.

*Cytotoxicity Assays.* For assays involving adherent cell lines, 250 cells were plated in triplicate in a 3 cm plate (Falcon) in 2−3 mL of drug-free Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% glutamine, and a 1% penicillin/streptomycin mixture. After 24 h, the drug-free medium was aspirated and replaced with

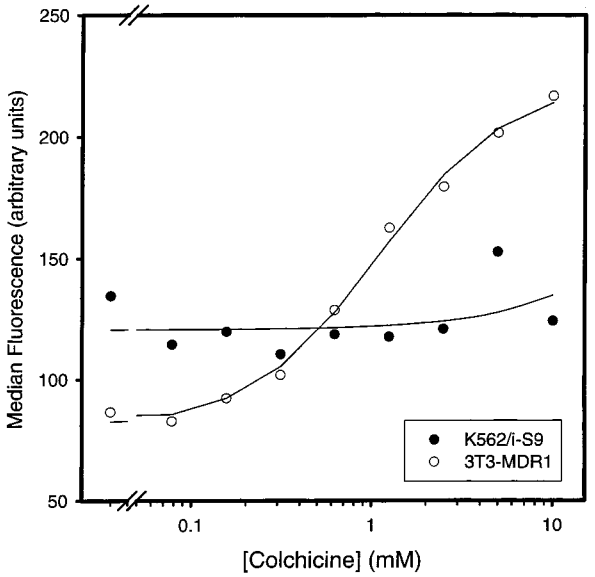


FIGURE 1: Effects of different concentrations of colchicine on UIC2 reactivity in K562/i-S9 and 3T3-MDR1 cells. Intact K562/i-S9 (●) and 3T3-MDR1 (○) cells were incubated at the indicated concentrations of colchicine for 10 min at 37 °C prior to the addition of UIC2 for an additional 30 min at 37 °C.

fresh medium containing the appropriate type and concentration of cytotoxic drug, in a 3 mL volume. After 8 days at 37 °C, the cells were fixed with methanol and stained with crystal violet (10% w/v in a 10% methanol solution) and the number of colonies per plate was counted.

For assays with suspension cell lines, 3 mL of medium containing 50 000 cells/mL and the appropriate concentration of cytotoxic drug were plated in a 3 cm plate, in triplicate. The cells were left to incubate at 37 °C for 5−7 days. After this incubation, cell clumps were disrupted by repeated pipetting and suspended in Isoton II Electrolyte Solution (Coulter), and the cell number was determined using a Coulter Z1 particle counter.

## RESULTS

*Differential Effects of Colchicine on the UIC2 Reactivity of the Human MDR1 Pgp in Different Cell Lines*. Figure 1 depicts the effects of colchicine on the UIC2 reactivity of Pgp in two multidrug-resistant cell lines. Due to the large number of reaction conditions and controls necessary to determine accurate parameters of substrate interactions with Pgp, this figure shows the results of a single experiment, but is representative of multiple independent experiments demonstrating similar results (not shown). In agreement with our previous report (*10*), increasing concentrations of colchicine up to 10 mM had no effect on the UIC2 reactivity of the K562/i-S9 cell line, derived from human K562 leukemia cells after retroviral transduction of the human *MDR*1 gene (●). In contrast, 10 $\mu$M vinblastine increased the UIC2 reactivity of this cell line ~4-fold (*11*). Colchicine, however, induced a 3-fold, dose-dependent increase in UIC2 reactivity in mouse NIH 3T3 cells transduced with a human *MDR*1-expressing retrovirus [3T3-MDR1 (○)]. To ensure that this effect of colchicine was not due to the ethanol in which colchicine was solubilized, we analyzed the effect of increasing ethanol concentrations on the UIC2 reactivity of 3T3-MDR1 cells. Ethanol had no effect on the UIC2 reactivity up to a concentration of 4% and increased this reactivity only at ≥5% (data not shown). It could not therefore account for the colchicine-induced increase in UIC2 reactivity in our reaction mixtures, which did not contain >1% ethanol.

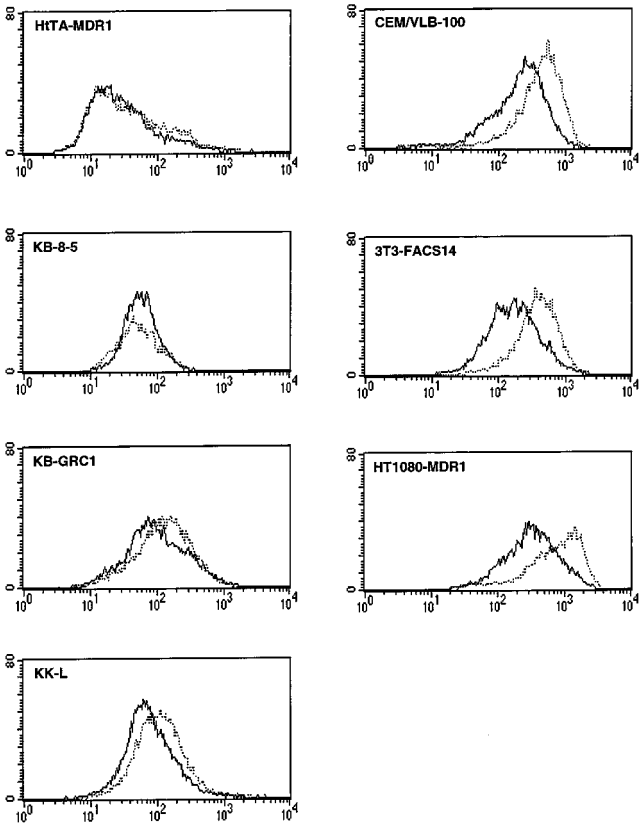


FIGURE 2: Effects of colchicine on UIC2 reactivity in different cell types. Each histogram depicts the fluorescence intensity of the indicated cell type, all expressing wild-type human Pgp, stained with UIC2 in the absence (—) or presence (···) of a saturating concentration (5−10 mM) of colchicine.

The solid line through the white circles in Figure 1 is the best-fit regression as determined using eq 1 in Experimental Procedures. From this regression, the $K_m$ value for the effect of colchicine on UIC2 reactivity in 3T3-MDR1 cells was 1.11 ± 0.14 mM. The $K_m$ values determined via UIC2 reactivity assays are apparent affinities of the ligand for Pgp and not the intrinsic affinity of the ligand for the protein. The Hill number ($n$) for the colchicine-induced increase in the UIC2 reactivity was 1.22 ± 0.19, which suggests that the binding of one molecule of colchicine to Pgp is sufficient to cause an increase in the UIC2 reactivity of 3T3-MDR1 cells. In contrast to colchicine, the Hill number for the increase in the UIC2 reactivity brought about by vinblastine was found to be close to 2 (*11*). These numbers are in agreement with the report that vinblastine has 2 (*18*, *20*) and colchicine only 1 binding site in Pgp (*19*).

Figure 2 demonstrates that a high concentration (5−10 mM) of colchicine has differing effects on UIC2 reactivity of Pgp in various other multidrug-resistant cell lines expressing the wild-type human MDR1 Pgp. Colchicine had little or no effect on the UIC2 reactivity of HeLa-derived HtTA-MDR1 cells and of two cell lines derived from the KB-3-1 cell line (also a subclone of HeLa) by colchicine selection (KB-8-5) or by transfection with the wild-type MDR1 cDNA (KB-GRC1). Colchicine produced a small increase in the

Table 1: Drug Resistance and Maximal Antibody Reactivity Values for Different Cell Lines Expressing the Wild-Type P-Glycoprotein[a]

| | $LD_{50}$ ($LD_{50}$ parental line) (nM) | | median fluorescence (arbitrary units) | | | | |
|---|---|---|---|---|---|---|---|
| cell line | vinblastine | colchicine | MRK16 | UIC2 | UIC2 and VLB | UIC2 and COL | UPC10 |
| K562/i-S9 | 73.0 (1.3) | 21.8 (3.6) | 588.35 | 125.21 | 604.30 | 119.71 | 4.45 |
| KB-GRC1 | 5.8 (0.3) | 5.0 (1.5) | 358.66 | 98.22 | 388.91 | 134.56 | 5.09 |
| CEM/VLB-100 | 150.6 (1.8) | 33.6 (1.6) | 552.32 | 220.67 | 661.17 | 449.10 | 3.72 |
| 3T3-MDR1 | 11.8 (0.9) | 144.2 (10.2) | 174.66 | 43.71 | 100.00 | 98.17 | 6.61 |
| HT1080-MDR1 | 3.7 (0.6) | 44.6 (3.3) | 947.46 | 278.81 | 905.80 | 835.36 | 24.14 |

[a] The derivations of all cell lines and their drug-sensitive parental lines are described in Experimental Procedures. The $LD_{50}$ values were determined from cytotoxicity assays. $LD_{50}$ values of the drug-resistant cell lines are followed by the $LD_{50}$ values for the drug-sensitive parental lines in parentheses. The median fluorescence (in arbitrary units) in the presence of saturating concentrations of MRK16 or UIC2 and in the absence or presence of a saturating concentration of either vinblastine or colchicine is listed.

UIC2 reactivity of human *MDR*1-transfected mouse LMtk$^-$ cells (KK-L), a stronger increase in a CEM leukemia cell line isolated by multistep selection with vinblastine (CEM/VLB-100), and an even stronger increase in the 3T3-FACS14 cell line, derived from NIH 3T3 cells by transduction with the human MDR1 retrovirus, and in human HT1080 fibrosarcoma cells transduced with MDR1 (HT1080-MDR1). In contrast to the variable effects of colchicine, saturating concentrations of vinblastine consistently increased UIC2 reactivity to a maximal level that was similar to the reactivity of a conformation-insensitive antibody MRK16 (Table 1) in almost all of these cell lines, with the exception of NIH 3T3 derivatives where the UIC2 reactivity in the presence of vinblastine remained lower than that of MRK16 (Table 1 and ref *14*).

*The Ability of Pgp To Confer Colchicine Resistance Depends on the Cellular Environment and Correlates with the Effect of Colchicine on UIC2 Reactivity*. We have previously shown that the levels of vinblastine resistance in MDR1-transduced NIH 3T3 cell lines correlate with the density of Pgp in the cell membrane (*20*). We asked if similar correlations could be established for vinblastine and colchicine in a comparison of different Pgp-expressing cell types, and if the ability of Pgp to confer resistance to different drugs could be related to the ability of such drugs to increase UIC2 reactivity. For this analysis, we measured the levels of vinblastine and colchicine resistance in five different drug-sensitive cell lines and in their multidrug-resistant derivatives, expressing the wild-type human MDR1 Pgp. Table 1 shows the $LD_{50}$ values for vinblastine and colchicine resistance for these multidrug-resistant cell lines and their drug-sensitive parents (in parentheses). Table 1 also includes the results of concurrent FACS assays for immunoreactivity (expressed as the mean fluorescence value) of the multidrug-resistant cell lines with a conformation-insensitive antibody MRK16 specific for the human MDR1 Pgp, with UIC2 in the absence or presence of vinblastine or colchicine, and with the UPC10 isotype control, the latter reflecting autofluorescence and nonspecific antibody binding (proportional to the cell surface).

Using the data in Table 1, we have plotted the relative resistance to vinblastine (Figure 3A) or colchicine (Figure 3B) against the relative density of Pgp in the individual cell lines. This latter parameter was determined by dividing the mean MRK16 immunofluorescence by the fluorescence of cells reacted with UPC10. The data show a strong correlation between vinblastine resistance and Pgp density, with a correlation coefficient ($r$) of 0.96 (Figure 3A). Similar results were obtained when UIC2 reactivity, in the presence or in the absence of vinblastine, was used in place of MRK16 reactivity to represent the Pgp levels (data not shown). The results depicted in Figure 3A indicate that the Pgp density is the primary determinant of vinblastine resistance in different Pgp-expressing cell lines, in agreement with the previous study, which was limited to the derivatives of a single cell line (*20*). In contrast to vinblastine resistance, however, colchicine resistance showed no correlation with Pgp density ($r = 0.12$; Figure 3B), indicating that some factors in the cellular environment other than the Pgp density affect the ability of Pgp to transport colchicine.

We asked if the ability of Pgp to confer colchicine resistance could be determined by its ability to undergo a conformational transition in the presence of colchicine. If this were the case, we would expect that relative colchicine resistance, normalized by relative vinblastine resistance (a function of the Pgp density), would show a Michaelis−Menten dependence on the colchicine-induced increase in UIC2 reactivity. Figure 3C shows a type of Lineweaver−Burke plot of the inverse values for the ratio of colchicine resistance to vinblastine resistance relative to the fold increase in UIC2 reactivity in the presence of saturating amounts of colchicine. This plot provides a linear regression with $r = 0.97$, indicating a highly significant correlation between the ability of colchicine to induce a UIC2 reactivity shift and the relative colchicine resistance conferred by Pgp in different cell types.

*Colchicine Counteracts the Vinblastine-Induced Increase in UIC2 Reactivity*. We investigated whether colchicine, which does not alter UIC2 reactivity in many of the tested cell lines, would affect the reactivity of such cell lines in the presence of a saturating concentration of vinblastine. Figure 4A shows the results of such an experiment with the KK-H cell line, which was derived from KK-L cells shown in Figure 2 by selection for increased resistance to vinblastine. Cells were reacted with increasing concentrations of colchicine, in the presence or absence of 10 $\mu$M vinblastine. While KK-H cells show a strong increase in UIC2 reactivity in the presence of vinblastine alone [compare the UIC2 reactivity for the starting point of the KK-H-labeled curve with the data point for KK-H cells at the same colchicine concentration ($\triangle$) in the absence of vinblastine], their UIC2 reactivity is not significantly affected by colchicine alone (compare the data points in the absence of vinblastine). In the presence of 10 $\mu$M vinblastine, however, increasing concentrations of colchicine steadily reduced the UIC2 reactivity of KK-H cells, bringing it close to the low level of reactivity observed in the presence of colchicine alone (Figure 4A). The best-fit regression using eq 2 in Experi-

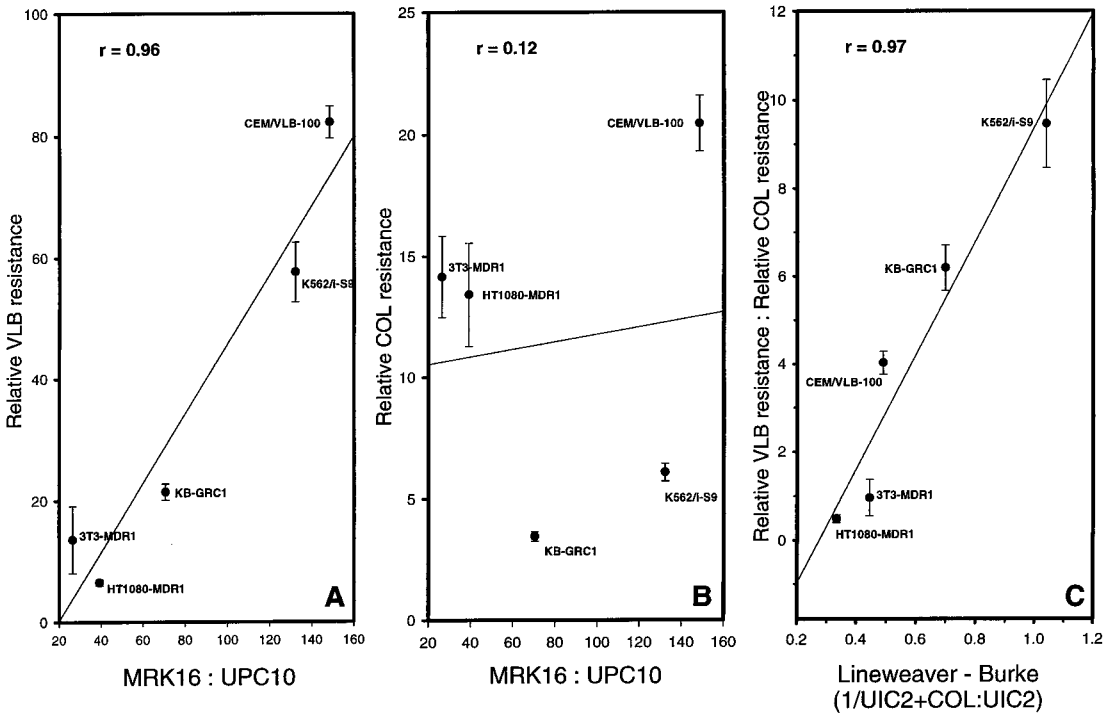


FIGURE 3: Correlations of vinblastine and colchicine resistance in different Pgp-expressing cell lines with Pgp density and UIC2 reactivity shift. Resistance and median fluorescence values for each of the five labeled cell lines are listed in Table 1. The solid line in each panel is the best-fit linear regression, and the error bars represent the standard error of the $LD_{50}$ ($n = 3$) as determined using SigmaPlot. In panel A, relative vinblastine (VLB) resistance (fold increase in $LD_{50}$ relative to that of the parental cell line) for each Pgp-expressing cell line is plotted on the *Y* axis vs the ratio of median MRK16 reactivity to median fluorescence of cells stained with the UPC10 control, a measure of Pgp density (*X* axis). In panel B, the relative colchicine resistance is plotted vs the same measure of Pgp density. Panel C is a type of Lineweaver−Burke plot for the ratio of median UIC2 reactivity to median UIC2 reactivity in the presence of a saturating concentration of COL (UIC2+COL) vs the ratio of relative VLB resistance to relative COL resistance.

mental Procedures shows that the $K_m$ of colchicine in the presence of vinblastine in KK-H cells was 6.68 ± 3.01 mM. The Hill number was 1.15 ± 0.34, suggesting that 1 molecule of colchicine was sufficient to decrease the UIC2 reactivity. Essentially the same effects of colchicine in the presence of a saturating concentration of vinblastine were obtained with K562/i-S9 cells, except that the $K_m$ was 1.96 ± 0.88 mM (data not shown). These results indicate that colchicine binds to Pgp and affects its conformation, even in the cells where colchicine alone does not bring about a change in the UIC2 reactivity. Furthermore, we have found that vinblastine, when tested up to a concentration of 72 $\mu$M, is unable to produce a significant increase in the UIC2 reactivity of K562/i-S9 cells in the presence of 5 mM colchicine (data not shown).

*Mutations of the Nucleotide-Binding Sites of Pgp Enhance the Effects of Colchicine on UIC2 Reactivity*. As described in the accompanying paper (*11*), the UIC2 reactivity of Pgp is affected by nucleotide binding and debinding. We asked if the ability of colchicine to change the UIC2 reactivity would be affected by mutations in the NBS of Pgp. Figure 4B compares the effects of increasing concentrations of colchicine on UIC2 reactivity of $LMtk^-$ cell lines transfected with either the wild-type human Pgp (KK-H) or Pgp mutants carrying K433M or K1076M substitutions of the essential lysine residues in the Walker A motifs of the N-terminal (MK-H) or C-terminal (KM-H) NBS, respectively. These mutants are capable of binding nucleotides but are devoid of ATPase activity (*5*), and their UIC2 reactivity is increased by vinblastine with the same Hill number (2) as the wild-type Pgp (*11*). As mentioned above, the UIC2 reactivity of the wild-type Pgp in KK-H cells was unaffected by colchicine (Figure 4B). Surprisingly, both cell lines carrying single NBS mutants of Pgp, KM-H (○) and MK-H (●), demonstrated a colchicine-dependent increase in UIC2 reactivity. The magnitude of this increase was higher for KM-H than for MK-H cells. The solid line through the data in Figure 4B is the best-fit regression using eq 1 in Experimental Procedures. The Hill numbers for the effect of colchicine on KM-H and MK-H cells were close to 1 (0.83 ± 0.50 and 1.44 ± 0.25, respectively). Since neither regression line reaches a saturation plateau for colchicine concentration, the apparent affinity of colchicine could not be measured from these data. These results indicate that colchicine can increase the UIC2 reactivity of Pgp mutants deficient in ATP hydrolysis, even in the cells where it does not alter the reactivity of the functional Pgp. We have previously shown that Pgp mutated in both NBS (MM) has a high UIC2 reactivity which is unchanged in the presence of vinblastine (*10*). As expected, colchicine also had no effect on the UIC2 reactivity of the MM mutant (not shown).

As with the wild-type KK-H cell line, increasing concentrations of colchicine decreased the UIC2 reactivity of KM-H and MK-H cells in the presence of 10 $\mu$M vinblastine to the levels approaching those that are seen with colchicine alone (Figure 4A). The Hill numbers for this effect of colchicine were close to 1 in all three cell lines (1.15 ± 0.34 for KK-H, 0.99 ± 0.46 for KM-H, and 1.00 ± 0.15 for MK-H cells). The apparent affinity for this effect of colchicine, however, was more than 1 order of magnitude higher for the single NBS mutants ($K_m$ = 0.28 ± 0.15 mM for KM-H and 0.23 ± 0.04 mM for MK-H cells) than in the wild-type Pgp of KK-H cells (6.68 ± 3.01 mM). These results provide

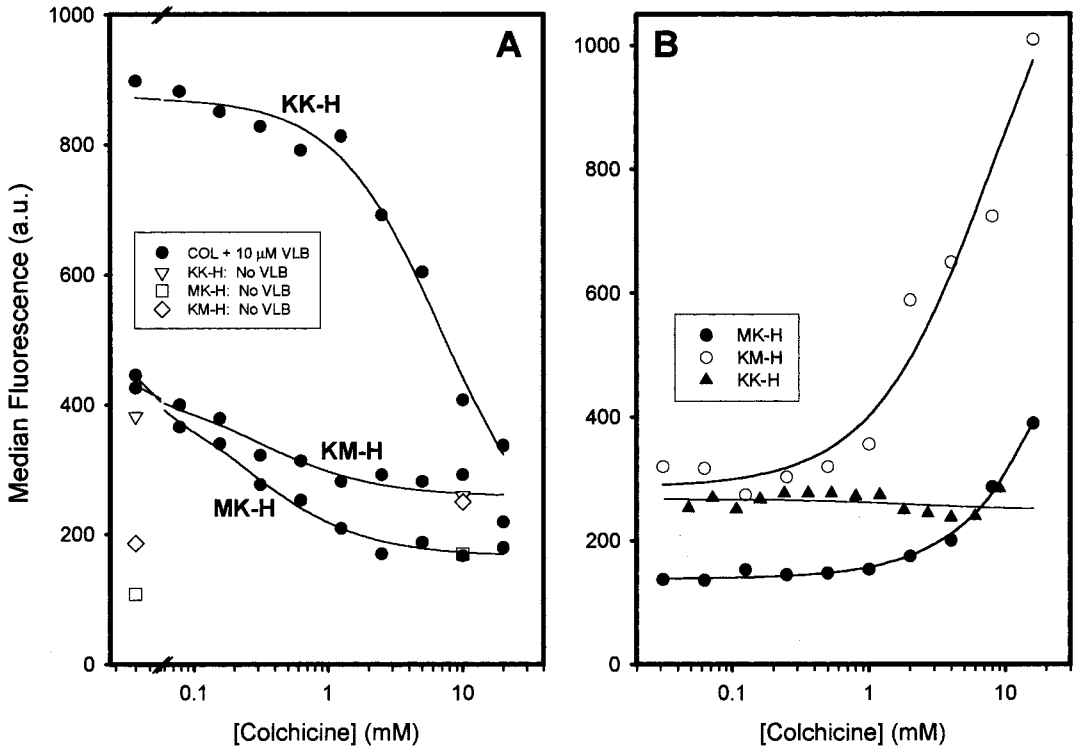


FIGURE 4: Effects of colchicine on UIC2 reactivity of the wild-type Pgp (A) and of ATPase-deficient Pgp mutants (B) in the presence of vinblastine. Both panels depict data for LMtk$^-$ cells expressing either wild-type Pgp, KK-H, or single-NBS mutants of Pgp, KM-H, and MK-H. In panel A, cells were incubated in the absence [KK-H (▽), KM-H (◇), and MK-H (□)] or presence of 10 $\mu$M vinblastine (●), individually labeled) for 10 min at 37 °C prior to the addition of the indicated concentration of colchicine. The cells were then stained with UIC2 for an additional 30 min at 37 °C. The solid line through each set of data is the best-fit regression as determined using eq 2. In panel B, KK-H (▲), KM-H (○), and MK-H (●) cells were stained at the indicated concentration of colchicine for 10 min at 37 °C prior to the addition of UIC2 and subsequent incubation for an additional 30 min at 37 °C.

additional evidence that mutations that abolish the ATPase activity of Pgp increase the effect of colchicine on UIC2 reactivity.

*Effects of Different Nucleotides on the UIC2 Reactivity in the Presence of Colchicine*. To investigate further the relationship between the conformational effects of colchicine and the nucleotide binding and hydrolysis by Pgp, we analyzed the effects of different nucleotides on the UIC2 reactivity in the presence of colchicine, using α-toxin-permeabilized cells. As described in the accompanying paper (*11*), cell permeabilization depletes cells of endogenous nucleotide, thereby increasing the UIC2 reactivity to the maximal level. The addition of ATP, ADP, or nonhydrolyzable ATP analogues decreases the UIC2 reactivity of permeabilized cells, but this effect is reversed by the addition of vinblastine (*11*).

Figure 5 shows the effects of ATP (panel A) or its nonhydrolyzable analogue AMP-PNP (panel B) on the UIC2 reactivity of permeabilized KK-H cells in the presence of nucleotide alone (●), nucleotide and 20 $\mu$M vinblastine (▼), or nucleotide and 10 mM colchicine (■). In agreement with our previous findings (*11*), both nucleotides provide a dose-dependent decrease in UIC2 reactivity in the absence of drugs, with the Hill number close to 2 for ATP (2.08 ± 0.42; Figure 5A) and closer to 1 (1.41 ± 0.21; Figure 5B) for AMP-PNP. Also as observed in the previous study (*11*), the presence of vinblastine (▼) reverses the decrease in UIC2 reactivity provided by either nucleotide alone (Figure 5A,B). The addition of the saturating concentration of colchicine, however, had a different effect in the presence of ATP than it had in the presence of AMP-PNP. In panel B, colchicine reverses the effect of the nonhydrolyzable analogue almost as efficiently as vinblastine. Colchicine was similarly effective in reversing the effect of ADP (data not shown). In contrast to its effect with nonhydrolyzable nucleotides, colchicine provides a moderate increase in UIC2 reactivity in the presence of lower concentrations of ATP, but at the highest ATP concentration (20 mM), the UIC2 reactivity is unaffected by the presence of colchicine (Figure 5A). An important distinction between the decrease in UIC2 reactivity provided by ATP alone versus ATP and colchicine is found when comparing the Hill number for each regression line. While the Hill number for ATP alone was approximately 2 (2.08 ± 0.42), the Hill number for ATP in the presence of colchicine was 0.60 ± 0.11 (which was not significantly different from 1). This result suggests that the number of ATP molecules required to decrease UIC2 reactivity is decreased in the presence of colchicine.

Similar experiments were carried out to analyze the UIC2 reactivity of single-mutant Pgps in permeabilized KM-H and MK-H cells. As shown in Figure 6, not only vinblastine but also colchicine was able to reverse the decrease in UIC2 reactivity provided by ATP. This effect of colchicine in single NBS mutants parallels its ability to increase the UIC2 reactivity of intact MK-H and KM-H cells (Figure 4B). As in intact cells, colchicine caused a smaller change in the UIC2 reactivity of MK-H than of KM-H cell line. Colchicine and vinblastine were also effective in reversing the effects of ADP and AMP-PNP in permeabilized MK-H and KM-H cells (not shown).

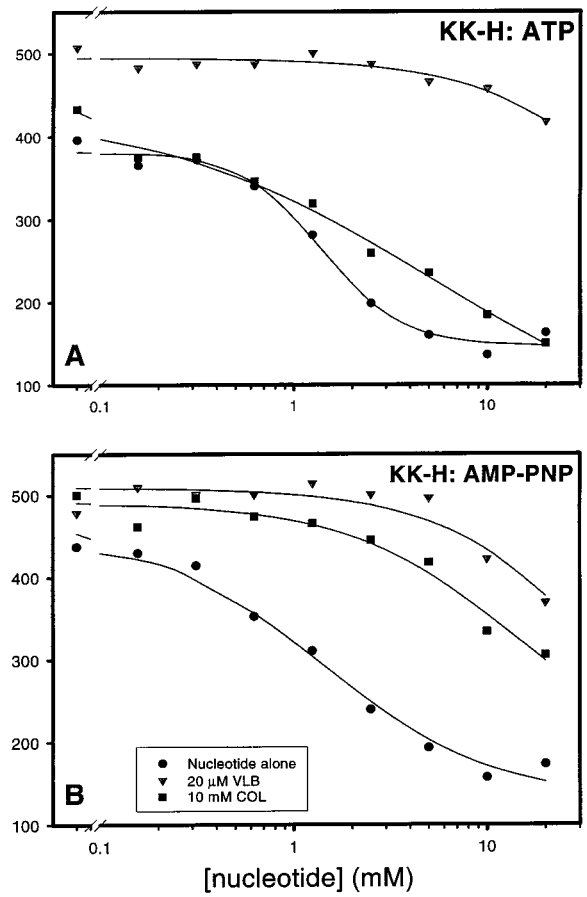


FIGURE 5: Effects of colchicine on the decrease in UIC2 reactivity of α-toxin-permeabilized cells brought about by ATP (A) or AMP-PNP (B). Both panels depict KK-H cells that were permeabilized with the α-toxin prior to the addition of nucleotide and/or drug. In each panel, circles (●) depict data for cells incubated in the presence of nucleotide alone (ATP in panel A or AMP-PNP in panel B), squares (■) depict data for cells incubated in the presence of nucleotide and 10 mM colchicine, and triangles (▼) depict data for cells incubated in the presence of nucleotide and 20 $\mu$M vinblastine. Cells treated with the α-toxin were incubated in the presence of nucleotide for 10 min at 37 °C followed by incubation for an additional 10 min at 37 °C after the addition of the appropriate drug. The solid line through each set of data is the best-fit regression as determined using eq 2.

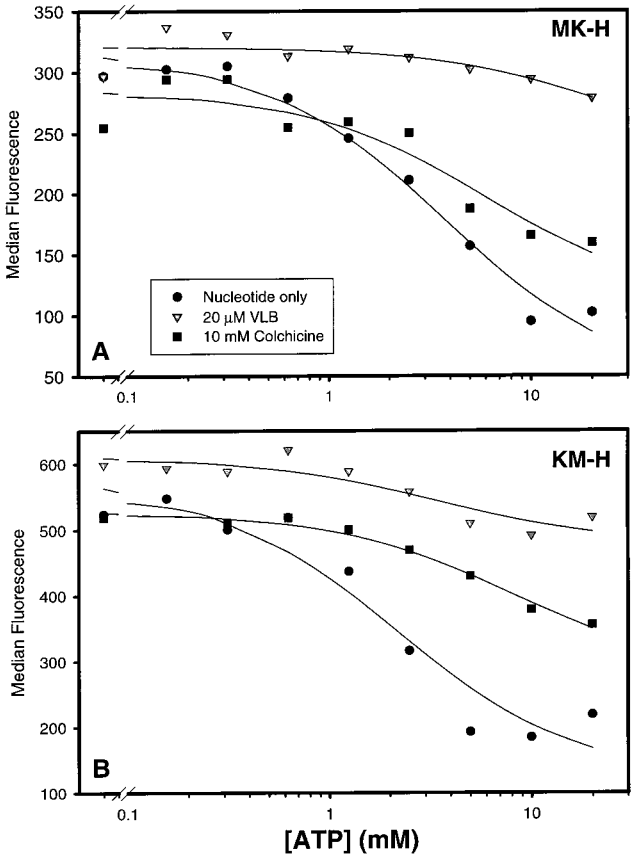


FIGURE 6: Effects of colchicine on the ATP-induced decrease in UIC2 reactivity in α-toxin-permeabilized MK-H (A) and KM-H (B) cells. In each panel, circles (●) depict data for cells incubated in the presence of ATP alone, squares (■) depict data for cells incubated in the presence of ATP and 10 mM colchicine, and triangles (▼) depict data for cells incubated in the presence of ATP and 20 $\mu$M vinblastine. The solid line through each set of data is the best-fit regression as determined using eq 2.

## DISCUSSION

In the study presented here, we compared the ability of Pgp expressed in different cell types to provide resistance to one of its relatively poor transport substrates, colchicine, and we have correlated this resistance with the ability of colchicine to induce conformational transitions of Pgp, which can be detected by altered reactivity with a conformation-sensitive antibody UIC2. We also compared how colchicine affects the Pgp conformation in the presence of another transport substrate, vinblastine, in the presence of ATP or nonhydrolyzable nucleotides, and in the wild-type Pgp relative to Pgp mutants deficient in ATP hydrolysis. We have found that the effects of colchicine on Pgp conformation depend on the cellular environment and on the ATP hydrolysis by Pgp and that these effects correlate with the ability of Pgp to confer colchicine resistance in different cell types.

Previous studies indicated that the relative resistance to different Pgp-transported drugs in the same cellular background depends on the specific Pgp isoforms (*21*) and on the presence of mutations that alter the substrate specificity of Pgp (*15*). In the study presented here, we asked if relative resistance to different drugs, conferred by the same wild-type human MDR1 Pgp, would be affected by the cell type where this Pgp is expressed. We have shown earlier that the resistance to vinblastine, one of the best transport substrates of Pgp, correlated with the cell-surface density of Pgp in different multidrug-resistant derivatives of the same cell line, suggesting that the Pgp level was the principal determinant of vinblastine resistance in such cells (*20*). We now extended this analysis to compare Pgp-expressing cell lines of different tissue types, and we still observed the same correlation between vinblastine resistance and Pgp density. In contrast, the relative levels of colchicine resistance varied widely and did not correlate with the Pgp density in different cell types. One possible interpretation of this variability was that some cell-specific mechanisms of colchicine resistance, unrelated to Pgp, determine the final level of colchicine resistance in Pgp-expressing cells. Alternatively, such cell-specific factors could still act through Pgp, by influencing its interactions with colchicine. Analysis of the effects of colchicine on the UIC2 reactivity shift in different cell lines supports the second interpretation. The levels of colchicine resistance relative to vinblastine resistance in different cell types showed an excellent correlation in a Lineweaver−Burke-type plot

with the ability of colchicine to induce UIC2-detectable conformational transitions. The differences in relative colchicine resistance or in the effects of colchicine on the Pgp conformation cannot be ascribed to the selection history of the cell lines analyzed in Figure 3. Three of the five lines were isolated without drug selection after retroviral transduction with wild-type human MDR1; one (KB-GRC1) was derived by transfection with a wild-type MDR1-expressing plasmid vector and a single step of small-scale colchicine selection to isolate the transfectants, and only one line (CEM/VLB-100) was derived by multistep selection with vinblastine, which has a potential for selecting additional mutations. The KB-GRC1 and CEM/VLB-100 lines, however, show no increase in the resistance to their selective agent relative to the Pgp density, which argues against selection-associated mutations in these cell lines. The differences in the colchicine response in different cell lines can therefore be attributed to the cellular environment rather than the effects of selection. To the best of our knowledge, this is the first demonstration that the cellular environment can alter the relative ability of Pgp to confer resistance to different drugs.

The finding that colchicine resistance is associated with colchicine-induced changes in the UIC2 reactivity was surprising, since colchicine (in contrast to vinblastine and most other Pgp substrates) was previously shown to be unable to bring about a change in UIC2 reactivity in K562/i-S9 cells (*10*). Similarly, colchicine was reported to induce no change in the proteolytic profile of Pgp, which is another assay for Pgp conformational transitions (*22*). By examining a variety of Pgp-expressing cell lines, we have now found that colchicine increases the UIC2 reactivity in some but not all cell lines. Furthermore, in those lines where colchicine alone did not affect UIC2 reactivity, this drug was able to reverse the vinblastine-induced increase in UIC2 reactivity. This result is consistent with the ability of colchicine to inhibit vinblastine-induced changes in the proteolytic profile of Pgp (*22*) and with the finding that high concentrations of colchicine inhibit vinblastine transport by Pgp (*23*). Our results suggest that the UIC2 reactivity shift may be used as an assay to identify Pgp-interacting agents by their ability to decrease the UIC2 reactivity in the presence of an upshifting substrate, such as vinblastine, even if such agents by themselves do not alter the UIC2 reactivity.

In those cell lines where colchicine induces an increase in UIC2 reactivity, the increase follows a conventional ligand-binding curve, which can be used to determine the apparent $K_m$ and the Hill number parameters that reflect Pgp−colchicine interactions. In all the cell lines analyzed in the study presented here, the Hill number for colchicine, determined in some lines by the increase in the UIC2 reactivity and in other lines by the decrease in UIC2 reactivity in the presence of vinblastine, was approximately 1, suggesting that the binding of only 1 molecule of colchicine is required to alter the UIC2 reactivity. This finding is consistent with other work reporting that colchicine has a single binding site on Pgp (*19*).

We have analyzed how the effects of colchicine on UIC2 reactivity are affected by nucleotide binding and ATP hydrolysis by Pgp. As we have previously shown (*11*), both ATP and nonhydrolyzable adenine nucleotides decrease the UIC2 reactivity in permeabilized cells, but vinblastine efficiently reverses this effect of nucleotides. Colchicine was also effective in reversing the effect of nonhydrolyzable nucleotides (AMP-PNP and ADP), suggesting that this substrate could also promote nucleotide dissociation. With ATP, however, colchicine was able to provide partial reversal of the UIC2 reactivity only at lower ATP concentrations. Since ATP, but not the nonhydrolyzable nucleotides, was able to overcome the conformational effect of colchicine, this result suggested that ATP hydrolysis could play a role in limiting the ability of colchicine to increase UIC2 reactivity. This hypothesis was confirmed in the assays using the KM-H and MK-H cell lines, which carry mutant forms of Pgp that bind, but do not hydrolyze, nucleotide (*5*). In contrast to cells carrying wild-type Pgp, colchicine increased the UIC2 reactivity of intact MK-H and KM-H cells and overcame the effects of the highest ATP concentrations in permeabilized cells.

Another important effect of colchicine on the Pgp−ATP interaction is indicated by the finding that the saturating concentration of colchicine (10 mM) altered the Hill number for the ability of ATP to decrease UIC2 reactivity from 2 to 1. This effect of colchicine may be interpreted in light of a recent hypothesis by Sauna and Ambudkar (*24*). They suggest that hydrolysis of 1 ATP molecule is required to transport the Pgp-bound substrate, with an associated change in the Pgp conformation [from E1 to E2, as depicted in the formal scheme given in Figure 6 of the accompanying paper (*11*)], that lowers substrate affinity. The hydrolysis of a second ATP molecule would change the Pgp conformation back to E1 to allow the transporter to bind a new substrate molecule. Colchicine binding appears to shift the equilibrium of these conformational transitions, in the direction from E2 to E1. In the absence of colchicine, such a shift in equilibrium would otherwise require the hydrolysis of a second ATP molecule. Therefore, in the case of colchicine, only one ATP would be required for the overall conformational transition, resulting in the decrease in the Hill number for ATP.

What is the nature of the cellular factors that determine the effects of colchicine on the Pgp conformation and the ability of Pgp to efflux colchicine? It seems likely that Pgp−colchicine interactions may be affected by the makeup of the lipid bilayer in the different cell lines. Several studies have reported that changes in the lipid composition of the plasma membrane alter drug and/or nucleotide binding to Pgp. Binding of the substrate [$^3$H]azidopine to reconstituted Pgp in liposomes was improved when the lipid composition of the liposomes was increased for cholesterol, stigmasterol, or ergosterol, in descending order (*25*). Also, [$^3$H]azidopine photolabeling of Pgp was abolished in the presence of nonionic detergents, but not in the presence of urea or a zwitterionic detergent, due presumably to disruption of the lipid bilayer (*26*). Similarly, alterations in the lipid headgroup and the acyl chain composition or the lipid bilayer alter the apparent affinities of vinblastine, verapamil, and daunorubicin for Pgp, and also affect ATP binding and hydrolysis (*9*).

It is also possible that Pgp interactions may be affected by some cytoplasmic factors other than lipid composition. Thus, Zhang and Ling (*27*) found that cytoplasmic components modulate the membrane topology of Pgp molecules produced in cell-free translation systems, and suggested that Pgp expressed in various cell types may have different topological structures. Such topological changes could account for the differences in proteolytic profiles of Pgp

observed in the presence of different ligands (*22*, *28*) and may provide a plausible explanation for altered UIC2 reactivity. The exact nature of substrate-induced conformational transitions of Pgp and the cellular factors that affect these transitions remain a subject for future investigation.

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BI001372N