# Isolation and Characterization of Caco-2 Subclones Expressing High Levels of Multidrug Resistance Protein Efflux Transporter

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***Purpose.*** The purpose of this study was to isolate Caco-2 subclones that express high levels of multidrug resistance protein (MDR1) and to characterize their kinetics and affinity parameters for MDR1 substrate/inhibitors.

***Methods.*** The subclones were selected by a dilution cloning technique. The polarized efflux of [$^3$H]-vinblastine across subclone cell monolayers was quantified by measuring the apparent permeability coefficients ($P_{app}$) of [$^3$H]-vinblastine in the basolateral (BL)-to-apical (AP) direction and in the AP-to-BL direction ($P_{app\ BL\text{-}to\text{-}AP}$/$P_{app\ AP\text{-}to\text{-}BL}$) across the cell monolayers. The expression of MDR1 in the Caco-2 subclones compared with the parental Caco-2 cells was confirmed by Western blotting analysis. The kinetics parameters ($K_m$, $V_{max}$) of [$^3$H]-vinblastine and the inhibitory constants ($K_I$) of several known MDR1 substrates/inhibitors on the transport of [$^3$H]-digoxin determined in the parental Caco-2 cells and Caco-2 subclones were also compared.

***Results.*** Three subclones (#1, #20, #21) were selected based on their polarized efflux of [$^3$H]-vinblastine. The $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios for #1, #20, and #21 were 110, 140, and 112, respectively, and were about 6-fold higher than the ratio observed for the parental Caco-2 cells. In the presence of GF-120918 (2 μM), a known MDR1-specific inhibitor, the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios were significantly decreased, suggesting that these cells were overexpressing MDR1. The $K_m$ values observed for vinblastine in the Caco-2 subclones were nearly identical to the value observed in the parental Caco-2 cells. In contrast, the $V_{max}$ values observed in the subclones were approximate 26–69% higher. The $K_I$ values observed for various known MDR1 substrates/inhibitors on [$^3$H]-digoxin transport were nearly identical to those in the parental Caco-2 cells and Caco-2 subclones. The high functional efflux activities of these subclones were stable up to 6 months.

***Conclusions.*** Subclones #1, #20, #21 express high levels of MDR1. These Caco-2 subclones may be useful models for profiling drugs for their MDR1 substrate activity and for establishing structure-transport relationships for this efflux transporter.

**KEY WORDS:** P-glycoprotein; subclone; Caco-2; efflux; inhibition; MDR1.

## INTRODUCTION

Caco-2 cell monolayers have become widely used as an *in vitro* model of the intestinal mucosa (1,2). Because the permeation characteristics of drugs across Caco-2 cell monolayers correlate with their human intestinal mucosa permeation characteristics, investigators have suggested that this cell line can be used to predict oral absorption of drugs in humans (3). However, this suggestion probably applies only to the drugs that permeate the human intestinal mucosa by passive diffusion and are not metabolized (4). Many drugs are substrates for transporters in the intestinal mucosa that can facilitate (e.g., peptide transporter; 5) or restrict (e.g., multidrug resistance protein [MDR1]; 6,7) their permeation. Some drugs are substrates for enzymes (e.g., cytochrome P450 3A4 [CYP3A4]) expressed in the human intestinal mucosa, and their intestinal permeation can be significantly restricted by the resulting metabolism (8).

Recently, intestinal MDR1 and CYP3A4 have been recognized as major determinates of oral drug bioavailability of some drugs (9). MDR1 was first characterized in cancer cells as the protein responsible for drug resistance (10). In the middle 1990s, MDR1 was also found to be widely expressed in normal tissues, such as the gastrointestinal mucosa, liver, pancreas, brain, and kidney (11). MDR1 was shown to be localized on the apical side of polarized cell membranes, such as the human intestinal mucosa (12). This polarized expression of MDR1 suggests that it functions as a secretory detoxifying system; thus, it represents a major barrier impeding permeation of some drugs across the intestinal mucosa (8). Therefore, cell lines that express "normal" and constant levels of MDR1 and/or CYP3A4 could become useful *in vitro* models to profile the substrate activities of a drug for one or both of these proteins or for mechanistic studies designed to evaluate the pathway by which a drug permeates the intestinal mucosa.

Caco-2 cells have been previously shown to express insignificant levels of CYP3A4 (13). In attempts to generate Caco-2 cells that express high levels of CYP3A4, 1α,25-dihydroxy vitamin-$D_3$ has been added to the growth medium to upregulate expression of this enzyme (14,15) and Caco-2 cells were transfected with the gene for CYP3A4 (16).

Caco-2 cells have been reported to express MDR1 (12,17,18) and to exhibit polarized efflux of MDR1 substrates (19). Based on these observations, Caco-2 cells have been used to quantitatively predict the human small intestinal efflux of drugs (1). However, the expression levels of MDR1 in Caco-2 cells have been shown to vary from laboratory to laboratory and with the way in which the cells are manipulated (2,20). Therefore, the data for the same substrate may be very different among laboratories as well as within the same laboratory. On the other hand, Caco-2 cells have also been found to express multidrug resistance-related proteins (e.g., MRP2; 18). The expression of multiple transporters in this cell model makes it difficult to design studies to elucidate the structure-transport relationship for individual transporters. Therefore, a Caco-2 cell line that expresses high level of MDR1 would be of great importance for drug screening purposes.

The objectives of this study were to isolate Caco-2 subclones that express high levels of MDR1 and to characterize their kinetics and affinity parameters for MDR1 substrate/inhibitors.

## MATERIALS AND METHODS

### Materials

Parental Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cloning

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discs were purchased from Fisher Scientific (Pittsburgh, PA, USA). MDCK cells transfected with the human *MDR1* gene (MDCK-MDR1) were a gift from Professor Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). GF-120918 was donated by Dr. Kenneth Brouwer (Glaxo-SmithKline, Research Triangle Park, NC, USA). Dulbecco's phosphate-buffered saline, Hanks' balanced salt solution (HBSS) (modified), cyclosporin A, reserpine, vinblastine sulfate, quinidine, verapamil, phenylmethylsulfonyl fluoride, and anti-rabbit IgG-horse radish peroxidase were purchased from Sigma Chemical (St. Louis, MO, USA). L-Glutamine (200 mM), penicillin (10,000 U/mL), streptomycin (10,000 μg/mL), and nonessential amino acids (10 mM [100×] in 85% saline) were obtained from Gibco BRL, Life Technologies (Grand Island, NY, USA). Dulbecco's modified Eagle medium and trypsin/EDTA solution (0.25% and 0.02%, respectively, in $Ca^{+2}$- and $Mg^{+2}$-free HBSS) were obtained from JRH Bioscience (Lenexa, KS, USA). Rat-tail collagen (type I) was obtained from Collaborative Biomedical Products (Bedford, MA, USA). Fetal bovine serum was obtained from Atlanta Biologicals (Norcross, GA, USA). Polycarbonate Transwells® (3-μm pore size) and polyester Transwells (0.4-μm pore size) were obtained from Corning Costar (Cambridge, MA, USA). [$^{14}$C]-Mannitol (specific activity 51 mCi/mmol) and [$^{3}$H]-digoxin (specific activity 19 Ci/mmol) were purchased from NEN Life Science Products, (Boston, MA, USA). [$^{3}$H]-Vinblastine sulfate (specific activity 11.1 Ci/mmol) was obtained from Amersham Life Science (Arlington Heights, IL, USA). Protease inhibitor cocktail (components: pancreas-extract, pronase, thermolysin, chymotrypsin, and papain) was obtained from Boehringer Mannheim (Germany). Molecular weight markers, 6% Tris-glycine gels, nitrocellulose membrane (0.45-μm pore side), and sodium dodecyl sulfate sample loading buffer were purchased from Invitrogen (Carlsbad, CA, USA). The bicinchoninic acid protein assay reagent kit was obtained from Pierce Chemical (Rockford, IL, USA). The primary antibody mdr Ab-1 was purchased from Oncogene Science (Cambridge, MA, USA). Electrochemiluminescence Western blotting detection reagents were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

### Cell Culture

Parental Caco-2 cells and its subclones were seeded at 79,000 cells/cm$^2$ on Transwell® filter inserts (6-well) or 100-mm dishes (for CYP3A4 analysis; 21) and maintained in high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 2 mM L-glutamine solution, 100 U/mL penicillin, and 100 μg/mL streptomycin as described previously (22). Cells (passages 20–40) were used on days 21–28 post seeding. To check the stability of Caco-2 subclones, the subclones were continuously cultured at conditions as described above (no toxic drug pressure) for 6 months.

MDCK-MDR1 cells were seeded at 50,000 cells/cm$^2$ on Transwell filter inserts (6-well) and maintained under the same conditions as described above for Caco-2 cells. Cells were used on day 6 postseeding. The expression of the MDR1 transporter in the MDCK-MDR1 cells was checked using Western blotting as described below. The MDCK-MDR1 cells were split twice a week with 1:10 dilution and used within 2 months after being removed from liquid $N_2$.

All cell lines were maintained at 37°C in a humidified 5% $CO_2$, 95% air atmosphere. Because all test compounds used in this study were [$^{3}$H]-labeled, it was possible to analyze the transport of the test compound and simultaneously monitor the cell membrane integrity by monitoring the permeation of [$^{14}$C]-mannitol. The apparent permeability coefficients ($P_{app}$) of [$^{14}$C]-mannitol across parental Caco-2, Caco-2 subclones, or MDCK cell monolayers were typically 0.1–0.72 × $10^{-6}$ cm/s.

### Isolation of Caco-2 Subclones

Parental Caco-2 cells (passage number 35) were plated at 100 cells/100-mm dish and the cells were allowed to grow into clones. After cells adhered to the dishes, the single cells were marked and cultured for 2–4 weeks. Individual clones were transferred to 24-well tissue culture plates using cloning discs saturated with 0.25% trypsin solution containing 0.02% EDTA. Individual clones were stepwise expanded to 6-well tissue culture plates, 25-mL tissue culture flasks, and 75-mL tissue culture flasks. The colony-forming efficiency of each clone was checked. Only the clones with colony-forming efficiency greater than 10% were selected for further transport studies (23).

### Transport Experiments

Bidirectional transport experiments were performed as previously described (22) with some modifications. All experiments were done at 37°C in air with constant mixing in a shaking water bath (60 rpm). Briefly, both the apical (AP) and the basolateral (BL) chambers of each insert were washed twice with 37°C HBSS for 15 min. Test drugs ([$^{3}$H]-digoxin, [$^{3}$H]-vinblastine, or [$^{14}$C]-mannitol at a concentration of 0.011 μM) were added to the donor side (1.5 mL for the AP chamber and 2.5 mL for the BL chamber) and fresh HBSS was placed in the receiver compartment. To inhibit the MDR1 efflux activity, cell monolayers were incubated with HBSS containing GF-120918 (2 μM) for an additional 15 min. The test compounds were added to the donor side along with GF-120918 (2 μM) and fresh HBSS containing GF-120918 (2 μM) was placed in the receiver side. Aliquots (100 μL) were withdrawn from the receiver side at various time to 80 min. Fresh HBSS or HBSS containing inhibitor (100 μL) was replaced in the receiver side after sampling.

$P_{app}$ values were calculated using the following equation:

$$P_{app} = \Delta Q/\Delta t/(A*C_0) \quad (1)$$

where $\Delta Q/\Delta t$ is the linear appearance rate of mass in the receiver solution, A is the filter/cell surface area (4.71 cm$^2$), and $C_0$ is the initial concentration of the test compounds. The net efflux of a test compound was assessed by calculating the ratio of $P_{app}$ in the BL-to-AP direction vs. $P_{app}$ in the AP-to-BL direction ($P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$).

### Western Blotting and Protein Detection

Western blotting was performed using a modification of the procedure described by Hosoya *et al.* (17). Confluent cell monolayers of parental Caco-2, Caco-2 subclone cells grown in 150-cm$^2$ flasks were collected and lysed in freshly prepared lysis buffer (1% TX-100 [v/v], 20 mM Tris HCl, 150 mM NaCl, 1:1000 protease inhibitor cocktail, 1 mM PMSF, and 1 mM

EDTA). The cell lysates were centrifuged and the supernatants containing the solublized protein were collected. The total cellular protein content was determined using the BCA assay. Aliquots (5 μg) of protein were size-fractionated in a 6% Tris-glycine acrylamide gel in 0.1% sodium dodocyl sulfate Tris-glycine buffer. Proteins were then transferred onto nitrocellulose membranes. MDR1 was probed using the primary antibody mdr Ab-1 (1:20 dilution) and the secondary antibody anti-rabbit IgG horseradism peroxidase (1:6000 dilution). Antibody detection was performed using the electrochemiluminescence method after the protocol provided by the manufacturer.

### Determination of Apparent Michaelis-Menten Constants ($K_m$, $V_{max}$) of [$^3$H]-Vinblastine in Parental Caco-2 and Caco-2 Subclone Cells

The time courses for the total flux rates of [$^3$H]-vinblastine in the BL-to-AP direction across parental Caco-2 and Caco-2 subclone cells were determined by sampling at 20-min intervals. The concentration dependence of the flux rate of [$^3$H]-vinblastine was also determined in the concentration range of 0.01–150 μM. The passive diffusion flux rate (excluding the influence of efflux transporter) of [$^3$H]-vinblastine was estimated by conducting the transport experiment in the presence of CsA (25 μM). The active transport flux rates were then estimated by subtracting the passive diffusion flux rates from total flux rates. Michaelis-Menten parameters were estimated using Origin 6.0 (OriginLab, Northampton, MA, USA).

### Determination of the Inhibitory Constants of MDR1 Substrates/Inhibitors on the MDR1-Mediated BL-to-AP Transport of [$^3$H]-Digoxin across Parental Caco-2 and Caco-2 Subclone Cell Monolayers

Apparent inhibition constants ($K_I$) of known substrates/inhibitors of MDR1 on the MDR1-mediated BL-to-AP transport of [$^3$H]-digoxin across Caco-2 and subclone cell monolayers were measured and calculated using the method previously published by Gao *et al.* (24). Working equations (2–4) used in these calculations were as follows:

$$K_I = [(P_I/P_0)/(1 - P_I/P_0)]*[I] \quad (2)$$

$$P_0 = P_{app,\,1} - P_{app,\,3} \quad (3)$$

$$P_I = P_{app,\,2} - P_{app,\,3} \quad (4)$$

In Eq. (2), $K_I$ is the apparent inhibition constant of a test compound. $P_I$ and $P_0$ are $P_{app}$ values of [$^3$H]-digoxin in the presence and the absence of the test compound, respectively. $P_I/P_0$ is a reflection of the inhibitory effect of the test compound on the active BL-to-AP transport of [$^3$H]-digoxin across the cell monolayers. [I] is the concentration of the test compound in the donor and the receiver side. $P_{app,\,1}$, $P_{app,\,2}$, and $P_{app,\,3}$ were determined from the BL-to-AP transport of [$^3$H]-digoxin in the absence of any compound, in the presence of a test compound, and in the presence of GF-120918 (2 μM), respectively. The active component of the permeation of [$^3$H]-digoxin in the absence of a potential inhibitor ($P_0$) and in the presence of a potential inhibitor ($P_I$) were determined by subtracting $P_{app,\,3}$ (the passive diffusion component) from $P_{app,\,1}$ (total transport in the absence of any compound), and $P_{app,\,2}$ (total transport in the presence of a potential inhibitor), respectively. To avoid cell-cell variations, the transport experiments for the determination of $P_{app,\,1}$, $P_{app,\,2}$, and $P_{app,\,3}$ were always conducted on the same day using the same batch of cells.

### Evaluation of the Expression Levels of CYP3A4 in Subclones

To evaluate the expression levels of CYP3A4 in the Caco-2 subclones, the cells were cultured under the same condition as described in the Cell Culture section. Western blotting analysis was performed using antibody of CYP3A4 as described in the Western Blotting and Protein Detection sections. The functional activity of CYP3A4 was measured by detecting the metabolism of testosterone by the Caco-2 subclone cells grown on 100-mm Petri dishes (21). The metabolites of testosterone was analyzed using high-performance liquid chromatography.

### Statistical Analysis

Statistical analyses were performed using Student's two-tailed *t* test between two mean values (Stat View 4.53, Abacus Concepts, Berkeley, CA, USA). A probability of less than 0.05 ($p < 0.05$) was considered to be statistically significant.

## RESULTS

### Selection of Caco-2 Subclones and Evaluation of Their Functional Efflux Activity

Twenty-one Caco-2 subclones were selected from the parental Caco-2 cells for further characterization. Among these subclones, fourteen had colony-forming efficiency greater than 10% (data not shown). These clones were further characterized as described below.

[$^3$H]-Vinblastine, a known substrate for MDR1 and MRP2 (25), was chosen to quantify the functional efflux activities of the parental Caco-2 cells and Caco-2 subclones. GF-120918 (26,27) was used in these studies as a specific inhibitor of MDR1. As shown in Fig. 1 and Table I, the parental Caco-2 cells and all of the Caco-2 subclones exhibited strong polarized efflux of [$^3$H]-vinblastine (i.e., $P_{app\ BL\text{-}to\text{-}AP} >> P_{app\ AP\text{-}to\text{-}BL}$). The ratio of $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$, which is used here as an indicator of efflux activity, was 17.9 and 30

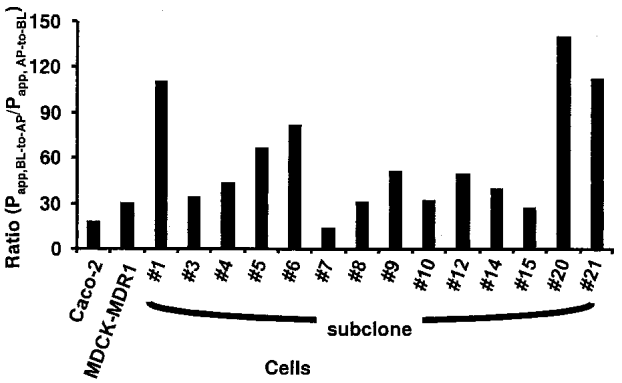


**Fig. 1.** The $P_{app,\ BL\text{-}to\text{-}AP}/P_{app,\ AP\text{-}to\text{-}BL}$ ratios of bi-directional transport of [$^3$H]-vinblastine across the parental Caco-2, MDCK-MDR1, and Caco-2 subclone cell monolayers. Values are the mean of triplicate samples.

**Table I.** Bidirectional Transport of [$^3$H]-Vinblastine across the Parental Caco-2, MDCK-MDR1, and Caco-2 Subclone Cell Monolayers

| Cell type | Treatment | $P_{app} \times 10^6$ (cm/s) AP-to-BL | $P_{app} \times 10^6$ (cm/s) BL-to-AP | Ratio $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ |
|---|---|---|---|---|
| Parental Caco-2 | — | 1.07 ± 0.04 | 19.2 ± 0.49 | 17.9 |
| | 2 μM GF[a] | 5.82 ± 0.21 | 9.75 ± 0.59 | 1.7 |
| MDCK-MDR1 | — | 0.40 ± 0.45 | 12.1 ± 0.54 | 30.3 |
| | 2 μM GF[a] | 0.67 ± 0.16 | 2.33 ± 0.37 | 3.5 |
| Subclone #1 | — | 0.30 ± 0.06 | 33.2 ± 1.16 | 110 |
| | 2 μM GF[a] | 3.55 ± 0.20 | 14.1 ± 0.64 | 4.0 |
| Subclone #20 | — | 0.21 ± 0.10 | 29.4 ± 1.27 | 140 |
| | 2 μM GF[a] | 5.29 ± 0.42 | 15.5 ± 0.89 | 2.9 |
| Subclone #21 | — | 0.29 ± 0.19 | 32.6 ± 0.10 | 112 |
| | 2 μM GF[a] | 3.86 ± 0.58 | 8.57 ± 0.35 | 2.2 |

[a] GF = GF-120918. $P_{app}$ values of [$^3$H]-vinblastine were measured in the absence or presence of inhibitor GF-120918 (2 μM) in various cell lines as described in Materials and Methods section. $P_{app}$ values are presented as mean of triplicate samples ± SD.

for parental Caco-2 cells and MDCK-MDR1 cells, respectively. In contrast, Caco-2 subclones #1, #20, and #21 had $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios of 110, 140, and 112, respectively (Table I). These ratios were about 6-fold higher than that of the parental Caco-2 cells and 4-fold higher than that of MDCK-MDR1 cells (Table I). In the presence of GF-120918 (2 μM), the ratios of $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ were significantly decreased to 4.0, 2.9, and 2.2 for #1, #20, and #21, respectively (Table I).

To compare the monolayer integrity of these Caco-2 subclones with that of the parental Caco-2 cell monolayers, the bi-directional transport of [$^{14}$C]-mannitol was studied. The $P_{app\ BL\text{-}to\text{-}AP}$ and $P_{app\ AP\text{-}to\text{-}BL}$ values of [$^{14}$C]-mannitol across the Caco-2 subclone cell monolayers were similar to the values for the parental Caco-2 cell monolayers and were in the range of 0.30–0.72 $\times 10^{-6}$ cm/s (data not shown).

### Western Blotting Analysis of MDR1 Expression in the Parental Caco-2 and Caco-2 Subclone Cells

To determine whether the increase in functional efflux activity of the Caco-2 subclones (i.e., #1, #20, and #21) resulted from increased expression levels of MDR1, the subclone cells were analyzed using Western blotting techniques. When cell homogenates of parental Caco-2 cells and cells of Caco-2 subclones #1, #20, and #21 were probed with the primary antibody mdrAb1 and the secondary antibody anti-rabbit IgG-HRP, all exhibited a single band at 170 kDa. In addition, the staining of this band for the cells of Caco-2 subclones #1, #20, and #21 was more intense than the staining observed for the parental Caco-2 cells (Fig. 2).

### Apparent Michaelis-Menten Constants ($K_m$, $V_{max}$) of [$^3$H]-Vinblastine in Parental Caco-2 and Caco-2 Subclone Cells

The kinetics parameters for the efflux of [$^3$H]-vinblastine in the parental Caco-2 cells and Caco-2 subclone cells were compared. As shown in Table II, the $K_m$ values in cells of subclones #1, #20, and #21 were similar to the value observed in the parental Caco-2 cells but different than the value observed in MDCK-MDR1 cells. The $V_{max}$ values in the Caco-2 subclone cells were greater than the value observed for the parental Caco-2 cells but similar to the value observed for MDCK-MDR1 cells (Table II).

### Inhibition Constants ($K_I$) of Known Substrates/inhibitors of MDR1 in Caco-2 Subclone and Parental Caco-2 Cells

The inhibitory effects of GF-120918, CsA, reserpine, vinblastine, quinidine, and verapamil on BL-to-AP transport of [$^3$H]-digoxin in the Caco-2 subclones were compared with the values observed in the parental Caco-2 cells and MDCK-MDR1 cells. As shown in Table III, the $K_I$ values of each compound observed in the Caco-2 subclones were very similar to the values observed in the parental Caco-2 cells. In contrast, significant differences were observed between the $K_I$ values observed in the Caco-2 subclones and those observed in MDCK-MDR1 cells (Table III).

### The Stability of the Functional Efflux Activity of Caco-2 Subclones

The stabilities of functional efflux activity of the Caco-2 subclones #1, #20, and #21 were monitored during storage and passage by measuring the bi-directional transport of [$^3$H]-vinblastine. The Caco-2 subclones were continuously cultured for 6 months and periodically assayed for their polarized transport of [$^3$H]-vinblastine. As shown in Fig. 3, the $P_{app\ AP\text{-}to\text{-}BL}$ values in Caco-2 subclones #1 and #20 increased slightly over the 6-month period; however, the $P_{app\ BL\text{-}to\text{-}AP}$/

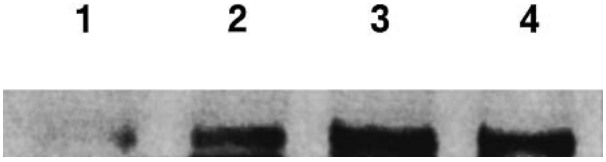


**Fig. 2.** Western blotting of MDR1 in total cell lysates from the parent Caco-2 and Caco-2 subclone cells. For each sample, 5 μg of total cellular protein was size fractionated in 6% Tris-glycine polyacrylamide gel in 0.1% of SDS Tris-glycine buffer. After electroblotting, MDR1 was stained with mdr Ab-1, and the protein-antibody interaction was visualized using the chemiluminescence technique as described in Materials and Methods section. Lane 1: parent Caco-2 cells; lane 2: subclone #1; lane 3: subclone #20; lane 4: subclone #21.

**Table II.** Apparent Michaelis-Menten Constants ($K_m$, $V_{max}$) for the MDR1-Mediated BL-to-AP Transport of [$^3$H]-Vinblastine across the Parental Caco-2, MDCK-MDR1, and Caco-2 Subclone Cell Monolayers

| | | | Caco-2 Subclones | | |
|---|---|---|---|---|---|
| | Parental Caco-2 | MDCK-MDR1 | #1 | #20 | #21 |
| $K_m$ (μM) | 89.2 ± 26.1 | 252.8 ± 134.7[a,b] | 90.1 ± 2.47 | 76.9 ± 31.9 | 81.0 ± 20.5 |
| $V_{max}$ (pmol · cm$^{-2}$ s$^{-1}$) | 1.77 ± 0.22 | 2.43 ± 0.86[b] | 2.23 ± 0.03[a] | 3.00 ± 0.49[a] | 2.65 ± 0.27[a] |

[a] Values were different from the values in the parental Caco-2 cells ($p < 0.05$).
[b] Data taken from Tang *et al.* (37).

$P_{app\ AP\text{-}to\text{-}BL}$ ratios remained significantly higher than the ratios observed with the parental cells (i.e., 77 and 81 for subclone #1 and #20, respectively). The transport of [$^3$H]-vinblastine in Caco-2 subclone #21 did not change over the 6-month period (Fig. 3).

Furthermore, the Caco-2 subclones also appeared to be quite stable to storage for up to 5 months in liquid $N_2$ (–180°C). For example, the $P_{app\ AP\text{-}to\text{-}BL}$ values and the $P_{app\ BL\text{-}to\text{-}AP}$ values of [$^3$H]-vinblastine in the Caco-2 subclones increased slightly; however, the ratios of $P_{app\ BL\text{-}to\text{-}AP}$/$P_{app\ AP\text{-}to\text{-}BL}$ remained higher than those of the parental Caco-2 cells (i.e., 102, 87, and 110 for #1, #20, and #21, respectively, Fig. 4).

### Evaluation of the Expression Levels of CYP3A4 in Subclones

When expression levels of CYP3A4 in the Caco-2 subclones and parental Caco-2 cells were estimated by Western blotting techniques, a slight increase in the staining of the CYP3A4 band was observed in the lysates of the subclone cells as compared with the cell lysates of the parental Caco-2 cells (data not shown). However, the functional activity of CYP3A4 as measured by metabolism of testosterone (21) was extremely low in both the Caco-2 subclones and the parental Caco-2 cells, consistent with literature reports (16; data not shown).

## DISCUSSION

Since the early 1990s, Caco-2 cells have been widely used by the pharmaceutical industry to estimate the intestinal mucosa and the substrate activity for efflux transporters (e.g., MDR1) of drugs/drug candidates (3). However, Caco-2 cells have some disadvantages including: 1) the time required to maintain cell monolayers in culture for maximal MDR1 expression (21 to 28 days); and 2) the inability to distinguish between the efflux activity of MDR1 and the efflux activities of other efflux transport proteins present in these cells (e.g., MRP2) (6). Therefore, efforts have been made to identify cell culture models (e.g., MDCK cells) that require less time to culture and cell culture models (e.g., MDCK-MDR1 cells) that express high levels of specific transporters (e.g., MDR1/MRP2). In the late 1990s, an MDCK-MDR1 cell line was generated by transfecting the human *MDR1* gene into MDCK cells (28). MDCK-MDR1 cells have been reported to express a high level of MDR1 (29) and\have been used as a model of the human MDR1 in the intestinal mucosa (30,31).

However, it is important to point out that the efflux activity of MDR1 has been shown to be closely connected to its membrane environment (32). Other researchers (32,33) observed that a few tightly bound lipids were retained on MDR1 during a purification process and the depletion of these lipids resulted in complete loss of activity, as measured by the ability of the protein to hydrolyze ATP. Because Caco-2 cells and MDCK cells are derived from different animal species (human versus dog) and different tissues (colonic carcinoma vs. kidney), differences may exist in the lipid composition of their cell membranes (34). These differences in lipid composition may change substrate-transporter (e.g., MDR1) interactions (35), which could result in differences in substrate/inhibitor specificity (drug binding) and differences in the kinetics of substrate efflux (36). Indeed, differences have been reported in the kinetics parameters ($K_I$ values) of known substrates/inhibitors in the parental Caco-2 cells and MDCK-MDR1 cells (31,37). The differences in substrate/inhibitor-MDR1 interactions observed in our laboratory (37) caused us to ques-

**Table III.** $K_I$ Values of Known MDR1 Substrates/Inhibitors on the Active BL-to-AP Transport of [$^3$H]-Digoxin across the Parental Caco-2, MDCK-MDR1, and Caco-2 Subclone Cell Monolayers

| | $K_I$ (μM)[a] | | | | |
|---|---|---|---|---|---|
| | | | Subclones | | |
| Substrates/inhibitors (Concentration used) | Parent Caco-2 | MDCK-MDR1[b] | #1 | #20 | #21 |
| GF120918 (0.2 μM) | 0.04 ± 0.01 | 0.44 ± 0.03 | 0.02 ± 0.01 | 0.05 ± 0.04 | 0.06 ± 0.02 |
| CsA (0.5 μM) | 0.35 ± 0.04 | 2.18 ± 0.31 | 0.61 ± 0.20 | 1.57 ± 0.38 | 0.37 ± 0.15 |
| Reserpine (10 μM) | 2.31 ± 0.35 | 11.5 ± 0.45 | 1.98 ± 0.11 | 1.87 ± 0.34 | 3.11 ± 0.56 |
| Vinblastine (35 μM) | 15.0 ± 3.78 | 140 ± 55.7 | 8.25 ± 0.71 | 9.82 ± 1.01 | 21.8 ± 1.65 |
| Quinidine (5 μM) | 0.43 ± 0.12 | 8.59 ± 1.68 | 0.86 ± 0.29 | 0.64 ± 0.48 | 0.49 ± 0.16 |
| Verapamil (5 μM) | 0.88 ± 0.06 | 15.1 ± 0.41 | 0.74 ± 0.08 | 1.82 ± 0.36 | 0.54 ± 0.26 |

[a] Data are presented as mean of triplicate samples ± S.D.
[b] Data taken from Tang et al. (37).

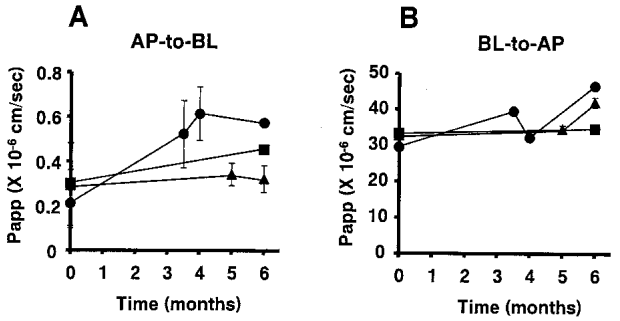


**Fig. 3.** The $P_{app\ AP\text{-}to\text{-}BL}$ (panel A) and $P_{app\ BL\text{-}to\text{-}AP}$ (panel B) of [$^3$H]-vinblastine in Caco-2 subclones continuously cultured for 6 months. ■, subclone #1; ●, subclone #20; ▲, subclone #21. Data are presented as mean of triplicate samples ± SD.

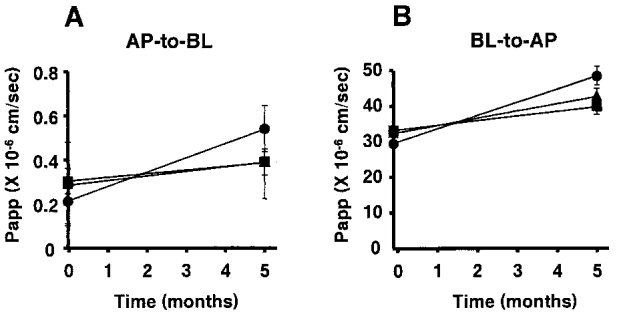


**Fig. 4.** The $P_{app\ AP\text{-}to\text{-}BL}$ (A) and $P_{app\ BL\text{-}to\text{-}AP}$ (B) of [$^3$H]-vinblastine in Caco-2 subclone cells before being frozen and after having been frozen in liquid $N_2$ for 5 months. ■, subclone #1; ●, subclone #20; ▲, subclone #21. Data are presented as mean of triplicate samples ± SD.

tion whether MDCK-MDR1 cells were good models of the intestinal mucosa. Therefore, our laboratory decided to attempt to develop subclones of Caco-2 cells that overexpress this efflux transporter.

Multidrug resistance cells can be selected as individual clones by growing single cells at a very dilute condition (100 cells/100-mm dish) or as mass populations by exposing the parental cells to toxic drugs and allowing the cells expressing low levels of efflux transporter to die back (23). If the second method were applied to the parental Caco-2 cells, a mass population that expresses multiple, different efflux transporters (e.g., MDR1, MRP2) might be isolated. Another disadvantage of using toxic drugs to isolate the subclones is that the selected cells usually need to be continuously cultured under toxic drug pressure to maintain high expression levels of efflux transporters. This constant exposure to the toxic drug pressure can cause alteration in cell membranes (36). A third method of developing multidrug resistance cells is to transfect the multidrug resistance gene into a cell line (25,38). Although a few animal cell lines transfected with the human multidrug resistance genes (e.g., MDCK-MDR1) have been reported (25,38), Caco-2 cells transfected with the MDR1 gene have not been described in the literature. This could be due to the heterogeneous properties of Caco-2 cells (2) or their rapid loss of the transfected gene during subsequent culturing. For example, Crespi *et al.* (16) reported that Caco-2 cells transfected with the CYP3A4 gene progressively lost metabolic activity of this enzyme upon passage of the transfected cells ($t_{1/2}$ ≈4 weeks).

In the present study, individual clone isolation methodology was used to select Caco-2 subclones that express high levels of MDR1. Using this methodology, the selected individual clones are much more likely to have a single gene that is responsible for the drug resistance (23). On the other hand, since the individual subclones are selected under a toxic drug-free condition, the gene that is responsible for the drug resistance in the selected subclone is intrinsic not induced during the selection process. Therefore, the expression of the multidrug resistance protein in these subclones might be more stable than in those subclones produced by transfection or toxic drug selection processes.

The 21 subclones isolated from the parental Caco-2 cells using this dilution cloning strategy were found to be different in colony-forming efficiency, monolayer integrity, and functional levels of expression of MDR1. Seven of the 21 subclones grew slowly and appeared to form leaky monolayers as determined by measuring [$^{14}$C]-mannitol permeation. No further characterization of these seven subclones was conducted. Through the bidirectional transport experiments, differences in the polarized efflux of [$^3$H]-vinblastine were observed with the remaining 14 subclones (i.e., the ratios of $P_{app\ BL\text{-}to\text{-}AP}$/$P_{app\ AP\text{-}to\text{-}BL}$ were in a range of 12–140; Fig. 1). By studying the flux of [$^{14}$C]-mannitol, these subclones were shown to have intact tight junctions similar to those observed in the parental Caco-2 cells. The observation that inclusion of GF-120918 (2 μM), a potent and specific inhibitor for MDR1 (26,27), decreased the polarized efflux of [$^3$H]-vinblastine in all subclone cells [(i.e., the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios were decreased to less than 4.0 (data only shown for #1, #20, and #21 in Table I) indicated that MDR1 is the major transporter responsible for the polarized efflux of [$^3$H]-vinblastine in these Caco-2 subclones.

To check whether the Caco-2 subclones (i.e., #1, #20, and #21) exhibited MDR1 substrate kinetics characteristics similar to those of the parental Caco2-cells, values of Michaelis-Menten constants ($K_m$, $V_{max}$) for efflux of [$^3$H]-vinblastine were determined. It is interesting to note that the $K_m$ values for the polarized efflux of [$^3$H]-vinblastine in these Caco-2 subclones were similar to that of the parental Caco-2 cells, whereas the values were quite different from those determined in MDCK-MDR1 cells (Table II). These data suggest that the cell type (probably cell membrane compositions) may be an important factor in determining substrate kinetics parameters (e.g., $K_m$) (31,36,37). As expected, the $V_{max}$ values in these subclones were greater than the value observed in the parental Caco-2 cells (Table II). These $V_{max}$ values are consistent with the higher functional efflux activity of these subclones as measured for the polarized efflux of [$^3$H]-vinblastine and the greater expression levels of MDR1 as measured using the Western blotting technique.

The $K_I$ values of known MDR1 substrates/inhibitors were also determined in parental Caco-2 cells, Caco-2 subclones, and MDCK-MDR1 cells (Table III). In general, the $K_I$ values determined for individual compounds in Caco-2 subclones were similar to the values observed in the parental Caco-2 cells. In contrast, the $K_I$ value observed in MDCK-MDR1 cells was significantly higher than the values observed in the parental Caco-2 cells and the Caco-2 subclones (Table III). Because a $K_I$ value is the dissociation constant of the compound from MDR1, the inverse of the $K_I$ value reflects the apparent affinity of the compound for this transporter.

Therefore, the greater the value of $K_I$, the lower the apparent affinity of a substrate/inhibitor for this efflux transporter. The data shown in Table III indicated that MDR1 in the Caco-2 subclones retained the same affinity for substrates/inhibitors as the MDR1 in the parental Caco-2 cells.

The stability of the expression of a transporter such as MDR1 in a cell line is an important determinant of whether it would be useful as a drug screening model. After continuous culturing for 6 months, the [$^3$H]-vinblastine $P_{app\ BL\text{-}to\text{-}AP}$/$P_{app\ AP\text{-}to\text{-}BL}$ ratio in subclone #21 did not change significantly (Fig. 3). The $P_{app\ BL\text{-}to\text{-}AP}$/$P_{app\ AP\text{-}to\text{-}BL}$ ratios of subclones #1 and #20 decreased slightly to 77 and 81, respectively, but they were still significantly higher than the ratios observed in the parental Caco-2 cells. It is worth noting that the Caco-2 subclones were cultured in the same "normal" growth medium as the parental Caco-2 cells. Exposure to toxic drugs is not needed to maintain these high levels of expression of MDR1. It should also be pointed out that these normal culturing conditions will probably prevent the subclones from undergoing changes typically seen with exposure to toxic drugs (36). These Caco-2 subclones were also shown to be stable under normal storage condition (i.e., after 5 months stored at –180°C, the $P_{app\ BL\text{-}to\text{-}AP}$/$P_{app\ AP\text{-}to\text{-}BL}$ ratios were 102, 87, and 110 for #1, #20, and #21, respectively, Fig. 4). Based on the data shown in Figs. 3 and 4, subclone #21 appears to have the best overall stability of the functional efflux activity of [$^3$H]-vinblastine.

In summary, three Caco-2 subclones (#1, #20, and #21) having significantly elevated functional efflux activity of MDR1 have been isolated. The MDR1 present in these Caco-2 subclones retained similar affinities for substrates/inhibitors of this efflux transporter. The functional activity of subclone #21 did not change after continuously culturing the cells for 6 months or storing the cells in liquid $N_2$ for 5 months. Therefore, this Caco-2 subclone may be a useful cell culture model for mechanistic studies designed to evaluate the role of MDR1 in modulating the permeation of drug/drug candidates or for studies designed to determine structure-transport relationships for this transporter.

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