molecular pharmaceutics

ACS AuthorChoice

Article

pubs.acs.org/molecularpharmaceutics

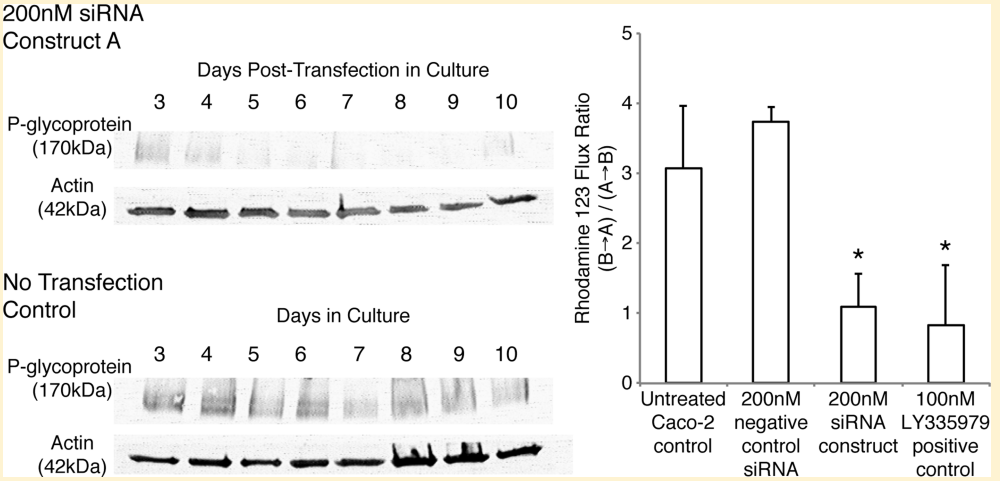
Terms of Use

Dose-Dependent Targeted Suppression of P-glycoprotein Expression and Function in Caco-2 Cells

Stephen D. Lee, Jo-Ann Osei-Twum, and Kishor M. Wasan*

Faculty of Pharmaceutical Sciences, University of British Columbia, 2405 Wesbrook Mall, Vancouver V6T 1Z3, British Columbia, Canada

Supporting Information



ABSTRACT: The efflux transporter P-glycoprotein (Pgp), encoded by the *ABCB1* gene, decreases the bioavailability of a wide range of orally administered drugs. Drug permeability studies using the in vitro Caco-2 cell model commonly rely on small molecule modulators to estimate the contribution of Pgp to drug efflux. The use of such modulators may be limited by their interactions with other membrane transporters. RNA interference, a tool allowing for the specific degradation of a target gene's mRNA, has emerged as a technique to study gene expression and function. This manuscript describes the use of chemically modified small interfering RNA (siRNA) for a dose-dependent suppression of *ABCB1* in Caco-2 cells and the subsequent drug permeability assay. We transfected Caco-2 cells while in suspension with chemically modified synthetic siRNA–lipid complexes and then seeded the cells on polycarbonate semipermeable supports. Once the monolayer of Caco-2 cells formed tight junctions and expressed brush border enzymes, we determined the dose-dependent suppression of the *ABCB1* gene using RT-qPCR. We measured the duration of silencing at the optimal siRNA dose by Western blot for Pgp protein. The utility of this in vitro model was determined by performing bidirectional transport studies using a well-established substrate for Pgp, rhodamine 123. A single 4 h transfection of the Caco-2 cells with ≥100 nM siRNA reduced the expression of *ABCB1* mRNA by >85% at day five in culture. The time-course study revealed that the single transfection reduces Pgp protein levels for 9 days in culture. This magnitude of silencing was sufficient to reduce the efflux of rhodamine 123 as measured by the apparent permeability coefficient and intracellular accumulation. In this study, we demonstrate the dose-dependent, targeted degradation of Pgp in Caco-2 cells as a new model for assessing drug efflux from enterocytes. The dose-dependent nature of the Pgp silencing in this study offers significant improvements over other approaches to creating a Caco-2 model with suppressed *ABCB1* expression. We envision that this technique, in conjunction with better small molecule inhibitors, will provide a useful tool for future drug permeability studies.

KEYWORDS: *drug transport, ATP-binding cassette transporters, P-glycoprotein, siRNA, gastrointestinal absorption, multidrug resistance*

INTRODUCTION

P-glycoprotein (Pgp), the membrane transporter encoded by the *ABCB1* gene (sometimes referred to as *MDR1*), is responsible for the efflux of a wide range of structurally diverse drugs from cells. Pgp is highly expressed in the cells of the small intestine, where it contributes to reduced bioavailability of

Received: November 21, 2012
Revised: March 31, 2013
Accepted: April 18, 2013
Published: April 18, 2013

drugs by limiting gastrointestinal absorption.[1] Pgp-mediated efflux can lead to drug−drug interactions, such as unpredictable bioavailability and increased clearance, when a patient is administered several medications concomitantly, including drugs that inhibit the activity or induce the expression of Pgp.[2,3] Consequently, new candidate drugs that are determined to be class II or class IV of the Biopharmaceutics Classification System[4] need to be screened early in the drug development process to determine if they are substrates for Pgp. This is most commonly achieved using cell culture transport studies in addition to preliminary in vitro screens. The Caco-2 cell line is the best-characterized model for the human small intestine because the cells differentiate into enterocyte-like cells that express brush border membrane enzymes.[5−7] Subsequent work showed that Caco-2 cells express Pgp and other membrane transporters at levels comparable to the human small intestine.[8] Caco-2 cells are typically grown on semiporous polycarbonate membranes for 14−21 days as a confluent monolayer that forms distinct basolateral and apical faces.[9] The contribution of Pgp to drug efflux of candidate compounds is measured by comparing the bidirectional transport of the drug across a monolayer of Caco-2 cells coincubated with established Pgp inhibitors to the transport across Caco-2 cells coincubated with vehicle controls.[9−11]

Effective Pgp inhibition is the subject of extensive cancer research programs because Pgp is often overexpressed in tumors where it contributes to multidrug resistance.[12,13] The first inhibitors of Pgp function were drugs developed against other targets that were found to reverse the Pgp-mediated efflux of cancer drugs in cell culture.[14] The most commonly cited first-generation inhibitors include progesterone, verapamil, and cyclosporine A.[15,16] Second-generation inhibitors were derivatives of first-generation inhibitors, such as the cyclosporine analogue valdospar (PSC-833), or they were discovered through chemical library screens, including elacridar (GF120918) and biricodar (VX-710). These compounds were designed for specificity to Pgp; however, extensive testing revealed that they induce cytochrome P450 drug detoxification enzymes and also inhibit other membrane-bound drug transporters.[14] Third-generation Pgp inhibitors such as zosuquidar (LY335979), tariquidar (XR-9576), and laniquidar (R101933) do not induce drug degradation pathways and have improved specificity but still exhibit some off-target inhibition of other membrane transporters.[14,15] To address these issues of specificity, a number of research groups have begun exploring RNA interference (RNAi) as a means to specifically decrease Pgp expression and function.[17−21]

The ability of small oligomers of RNA to specifically silence the expression of target genes was first observed in nematodes and has become a widely used tool for investigating gene function in experimental organisms.[22] Gene silencing is achieved through the binding of a 21−22bp RNA molecule to the complementary sequence on the target messenger RNA. An RNA induced silencing complex binds and degrades double-stranded RNA, thereby reducing the expression of the target gene. By using effective bioinformatics approaches, the small interfering RNA (siRNA) will only bind to a single target giving researchers a precise genetic tool to suppress gene expression and function.[23] Cells are generally transfected with a duplex of siRNA through the use of cationic lipid transfection reagents, resulting in a transient suppression of gene expression. The suppression of *ABCB1* expression in cancer cells by RNAi lasted only 48−72 h.[24] Differentiated epithelial cell lines such as Caco-2 require long culture times (14−21 days)[10,11] and are difficult to transfect;[25−27] consequently, standard transfection protocols[28] are not effective and other techniques are required. The most common approach is to create a stably transfected Caco-2 cell line with a plasmid encoding a small hairpin RNA (shRNA) sequence.[17−19,29] The plasmid also encodes a resistance gene for a toxic antibiotic, which allow researchers to select for Caco-2 cells that contain the plasmid by screening for resistance to the antibiotic. An alternate technique involves transduction of the cells with a retrovirus containing an siRNA sequence.[21] These approaches have several advantages including a stable suppression of gene expression over several passages and the ability to grow Caco-2 cells using standard techniques. Unfortunately, this technique does not allow researchers to titrate the dose of siRNA and the cells must be grown in the presence of aminoglycoside antibiotics to select for successful transfectants, which can activate the JNK stress pathways in vitro.[30]

Chemical modification of siRNA can improve the duration of silencing while reducing the nonspecific innate immune response associated with double-stranded RNA transfection. These improvements to siRNA might allow researchers to work with RNAi in cell lines that are good models for the small intestine, like Caco-2 cells grown on polycarbonate membranes.[31] In this paper, we test the utility of commercially available chemically modified siRNA to suppress Pgp expression in Caco-2 cells using a novel transfection approach. Our data support the hypothesis that RNAi can be used to suppress Pgp expression, and we show that Pgp function is decreased in a differentiated Caco-2 cell monolayer on semipermeable polycarbonate membranes.

EXPERIMENTAL SECTION

Reagents. Caco-2 cells (HTB-37, passage 17) were purchased from the American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle Medium (powdered high glucose without L-glutamine or $NaHCO_3$), L-glutamine, 100× penicillin−streptomycin (10000 U/mL, 10000 μg/mL), phosphate buffered saline (PBS), 0.25% Trypsin-50 mM EDTA, Stealth siRNA silencing vectors, lot-matched fetal bovine serum (qualified), TRIzol, Quant-iT RiboGreen RNA assay, Quant-iT OliGreen ssDNA assay, UltraPure DEPC-treated (RNase/DNase-free) water, Superscript III First Strand Synthesis SuperMix for qRT-PCR, TaqMan gene expression assays, ZO-1 (N-terminus) rabbit polyclonal antibody, goat antirabbit-HRP488 monoclonal antibody, DAPI (4′-6-diamidino-2-phenylindole dihydrochloride), and Prolong AntiFade Gold were purchased from Life Technologies (Carlsbad, CA). Sterile cell culture treated flasks (75 cm^2) and 12-well Transwell semipermeable supports (0.4 μm pore size) were purchased from Corning (Lowell, MA). Zosuquidar trihydrochloride was purchased from Cedar Lane Laboratories (Burlington, NC). HPLC-grade chloroform and 2-propanol were purchased from Thermo Fisher Scientific (Waltham, MA). siLentFect lipid transfection reagent and iTaq supermix with ROX were purchased from Bio-Rad (Hercules, CA). Triton X-100, $NaHCO_3$, protease inhibitor cocktail, phenylmethanesulfonyl fluoride, and bovine serum albumin (fatty acid free) were purchased from Sigma-Aldrich (St. Louis, MO). The lyophilized chemically modified Stealth siRNA was reconstituted in DEPC-treated water to a final concentration of 20 μM and stored in 100 μL aliquots at −20 °C.

Caco-2 Culture. Caco-2 cells (passage 25−38) were used for all experiments. Cells were grown on tissue culture treated 75 cm^2 flasks at 37 °C in a humidified 5% CO_2 incubator. The growth medium was Dulbecco's Modified Eagle Medium (DMEM) containing 1.5g/L $NaHCO_3$ (for a 5% CO_2 atmosphere) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The pH of the medium was adjusted to 7.3 and sterile filtered prior to use.

Transfection. The transfection conditions below were adapted from the protocol first published by Clayburgh et al.[32] Our modified protocol is summarized in Figure 1, and the

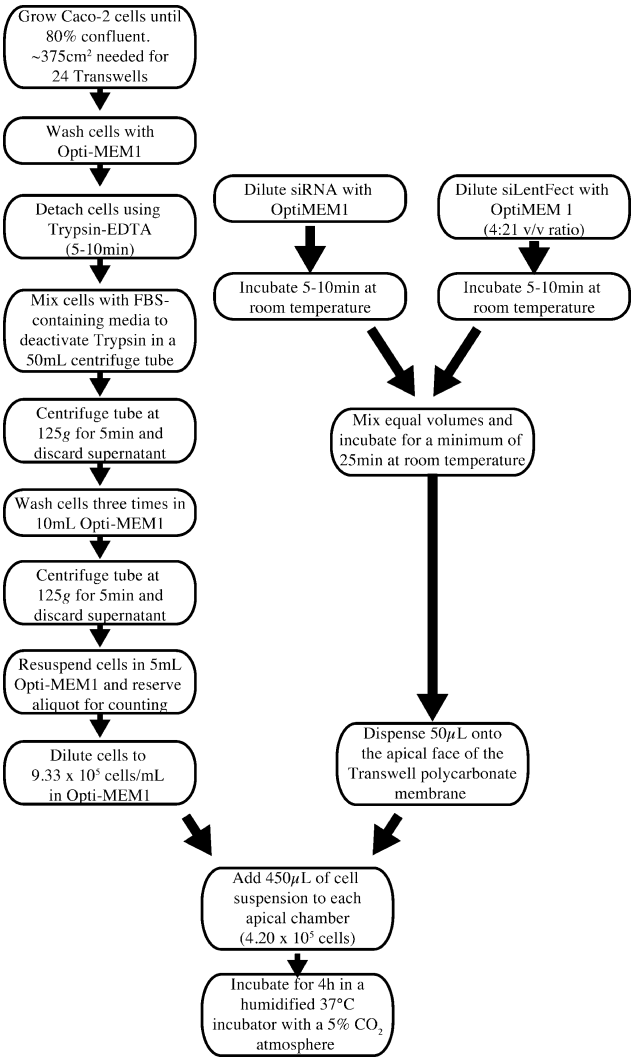


Figure 1. Summary of the transfection protocol.

siRNA sequence information is included in the Supporting Information. The transfection was set up so that the siRNA and siLentFect transfection mixture was 10% of the total volume (50 μL), while the cell suspension was 90% of the volume (450 μL). Initial optimization experiments varied the lipid transfection reagent content while maintaining a constant siRNA concentration. The second step in the optimization experiments was to establish a dose−response of each siRNA construct. The final transfection protocol is as follows: in a sterile polystyrene plate, siLentFect transfection reagent was diluted 4:21with Opti-MEM1. In a separate well, the siRNA construct was diluted from 20 μM stock to a concentration 20× higher than the final desired concentration. After 5−10 min of incubation at room temperature, equal volumes (30 μL per well needed) of diluted siLentFect and siRNA were mixed and incubated for a minimum of 25 min at room temperature. The cells from five tissue culture flasks (375 cm^2 total surface area) were washed with Opti-MEM1 and lifted from the flask using Trypsin-EDTA, 3 mL per flask for 5−10 min. The cells were transferred to a 50 mL centrifuge tube and mixed with DMEM growth medium to inactivate the trypsin. The tube was centrifuged at 125*g* for 5 min and the supernatant discarded. Washing the cell pellet three times with 10 mL of Opti-MEM1 media removed FBS and antibiotics. After the final washing step, the cell pellet was resuspended in 5 mL of Opti-MEM1 for counting prior to preparing a dilution of 933333 cells/mL in Opti-MEM1. In the apical chamber of a 12-well Transwell plate, 50 μL of transfection reagent was mixed with 450 μL of cell suspension (420000 cells). After filling the basolateral chambers with 1.5 mL of Opti-MEM1, the Transwell plate was incubated in a humidified 37 °C incubator with a 5% CO_2 atmosphere. After 4 h, the reagents were removed by aspiration and replaced with DMEM growth medium. Media was changed every 48 h until the end of the experiment.

RT-qPCR. The polycarbonate membranes was excised from the Transwell and placed in a well of a sterile 12-well polystyrene plate. Total RNA was extracted from the Caco-2 cells using 1 mL of TRIzol and a cell scraper. The TRIzol was transferred to a 2 mL polypropylene centrifuge tube, and the RNA was isolated as per the manufacturer's instructions. The RNA was quantified using the Quant-iT RiboGreen RNA Assay according to the manufacturer's instructions. Each RNA solution was diluted to a final concentration of 0.2 mg/mL with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). cDNA was prepared from RNA using the SuperScript III First Strand Synthesis SuperMix for RT-qPCR according to the manufacturer's instructions. cDNA was quantified using the Quant-iT OliGreen ssDNA assay according to the manufacturer's instructions. All samples were diluted to 10 ng/μL in DEPC-treated water. The expression of *ABCB1* was determined relative to untreated cells using TaqMan gene expression assays in an ABI 7900HT real time thermocycler (Life Technologies, Carlsbad, CA) with *ACTB* (β-actin) as the reference gene.

Formation of Tight Junctions. Transepithelial electrical resistance (TEER) was used to measure the formation of a monolayer of cells linked by tight junctions. The formation of tight junctions was qualitatively confirmed by examining the subcellular localization of the tight junction protein ZO-1 using immunofluorescence microscopy (details in Supporting Information).

Western Blot. Protein levels were qualitatively observed using Western Blot techniques. The polycarbonate membrane was excised from the Transwell and placed in a clean 12-well polystyrene plate. The cells were lysed in 0.25 mL of modified radioimmunoprecitation assay (RIPA) buffer containing protease inhibitors (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin). Aliquots of the lysate containing 20−30 μg of protein were separated by SDS-PAGE, transferred to nitrocellulose, and probed for Pgp, actin, and sucrase-isomaltase (details available in the Supporting Information)

Rhodamine 123 Uptake. Cells were seeded on Transwell semipermeable supports as described above. After 4 days, media was changed and untreated cells were preincubated with either 100nM LY335979 or 1% vehicle control (DMSO). On day 5 postseeding, TEER values were measured and cells were washed with transport buffer (Hanks Buffered Salt Solution). Cells were then incubated with 5 μM rhodamine 123 (Rh123), prepared in transport buffer, for 3 h in a humidified 37 °C incubator with a 5% CO_2 atmosphere. Rh123 was added to either the apical or basolateral face of the monolayer, with transport buffer in the corresponding receiver chamber. Untreated cells were coincubated with either 100 nM LY335979 or 1% vehicle control. After 3 h, cells were washed with transport buffer and then stored at −20 °C. To determine Rh123 uptake, polycarbonate membranes were excised from Transwell semipermeable supports and cells were lysed with 0.25 mL of cold modified RIPA. Rh123 concentrations were measured in 50 μL aliquots with a Fluoroskan Ascent fluorometer (Thermo Electron Corporation, Waltham, MA) (excitation = 485 nm and emission = 538 nm). Fluorescence values were normalized with respect to protein content.

Transmembrane Transport of Rhodamine 123. Cells were seeded on Transwell semipermeable supports as described above. On the fourth day post-transfection, the untreated cells were preincubated with either 100 nM LY335979 or 1% vehicle control. On day 5 postseeding, TEER values were measured and cells were washed with transport buffer. Cells were then incubated with 5 μM Rh123, prepared in transport buffer, for 2 h in a humidified 37 °C incubator with a 5% CO_2 atmosphere. Rh123 was added to either the apical or basolateral chamber, with transport buffer in the corresponding receiver chamber. Untreated cells were coincubated with either 100 nM LY335979 or vehicle control. Then 50 μL aliquots were sampled, with replacement, every 30 min from the receiver chamber. Rh123 concentrations were measured with a Fluoroskan Ascent fluorometer (excitation = 485 nm and emission = 538 nm). The apparent permeability coefficient and efflux ratio were calculated using the following equations:

$$P_{app} = \left(\frac{dQ}{dt}\right) \times \left(\frac{1}{A \times C_0}\right) \qquad \text{efflux ratio} = \frac{P_{app_{(B\to A)}}}{P_{app_{(A\to B)}}}$$

Data Analysis. All data sets were analyzed for statistical significance by parametric methods using GraphPad Prism (version 6.0). Data were analyzed for differences using one-way ANOVA with fixed effects. The groups were distinguished using Dunnet posthoc tests against the untransfected cells as a control. The gene expression data sets required an Ln-transform prior to analysis for the data to meet the requirements of parametric analyses. The mRNA data were analyzed by two-way ANOVA with Tukey posthoc tests. An alpha value of 0.05 was determined a priori for all statistical testing.

RESULTS

After a series of experiments determining the optimal conditions to transfect the Caco-2 cells, we were able to conduct siRNA dosage experiments using the protocol outlined in Figure 1. We conducted dose−response studies with three different *ABCB1*-targeting siRNA constructs to determine the minimum effective dose of each siRNA required to suppress *ABCB1* mRNA by >70%, as recommended for RNAi experiments,[33] five days post-transfection (siRNA sequences and alignment to the *ABCB1* mRNA are available in the Supporting Information). Five days post-transfection was selected as the time point because of the TEER values that were measured in Caco-2 monolayers in preliminary experiments. Each dose of siRNA was repeated with cells at two or three separate passage numbers to ensure the reproducibility of the results. The data from these studies are summarized in Figure 2. The dose−response studies revealed that a single dose

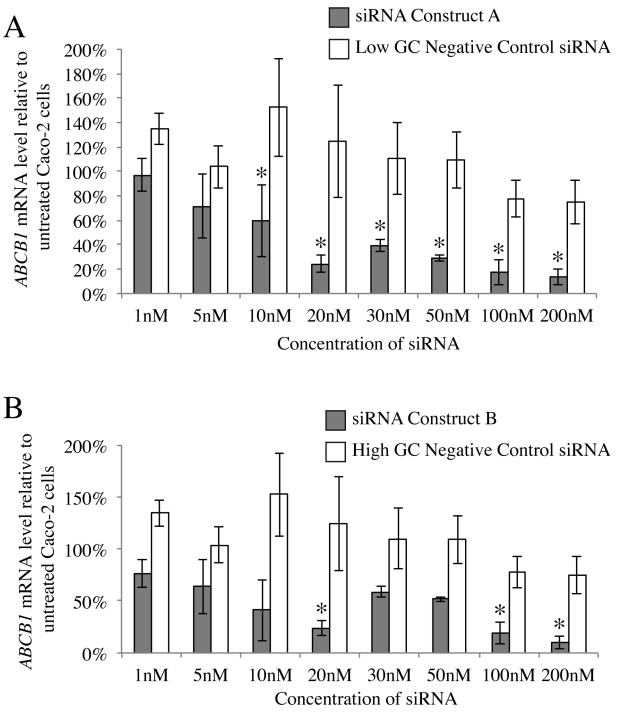


Figure 2. Dose response of the *ABCB1*-targeting siRNA constructs. The expression of *ABCB1* was determined by RT-qPCR for cells treated with one of two siRNA constructs, a GC-matched negative control, or media as an untreated control. The mean expression for the siRNA constructs ($\Delta\Delta Ct$ method versus the untreated control cells) at day 5 post-transfection is shown ± SD for 2−3 independent experiments. * $p < 0.05$ vs negative control siRNA by two-way ANOVA with Tukey posthoc tests.

of 100 nM of sequence A or 200 nM sequence B reduced *ABCB1* mRNA by >70% relative to untreated cells at five days post-transfection; however, the silencing was inconsistent when sequence C was used. Our experimental design included treatment with negative control siRNA sequences with similar GC content at each dose in each experiment to account for nonspecific transfection-related effects on *ABCB1* mRNA. We conducted Western blots to determine the length of time needed for cellular Pgp to be cleared post-transfection and to confirm that the single dose of siRNA decreased Pgp protein levels up to six days post-transfection, as shown in Figure 3. Pgp protein was detected in cell lysates for up to 96 h post-transfection, consistent with Pgp turnover (half-life of 5−17 h) reported in other cell lines.[34−36] To determine the maximum possible duration of silencing, we transfected Caco-2 cells with 200 nM siRNA (construct A or construct B) and plated the cells on polycarbonate membranes. Pgp protein levels were decreased until day 10 post-transfection relative to untransfected cells (Figure 4). Treating the cells with *ABCB1*-targeting siRNA had no effect on BCRP or MRP2 protein levels

dx.doi.org/10.1021/mp300668e | *Mol. Pharmaceutics* 2013, 10, 2323−2330

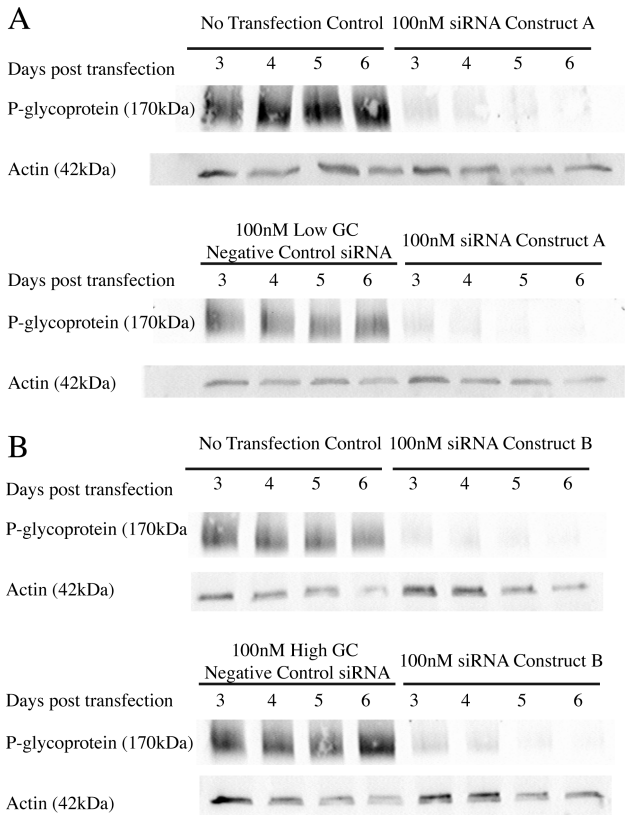


Figure 3. Effect of siRNA transfection on Pgp protein levels. The Pgp level in Caco-2 cells treated with construct A, construct B, a GC-matched control siRNA, or media alone was determined using Western blot at days 3, 4, 5, and 6 postseeding on polycarbonate membranes. β-Actin was measured as a loading control for each blot.

(Supporting Information Figure 1), confirming the specificity of this approach.

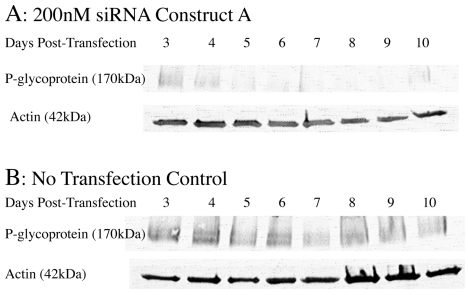


Figure 4. Duration of Pgp silencing with a single transfection of siRNA. The Pgp level in Caco-2 cells treated with 200 nM of construct A or untransfected Caco-2 cells was determined by Western blot at days 3−10 postseeding on polycarbonate membranes. β-Actin was measured as a loading control for each blot.

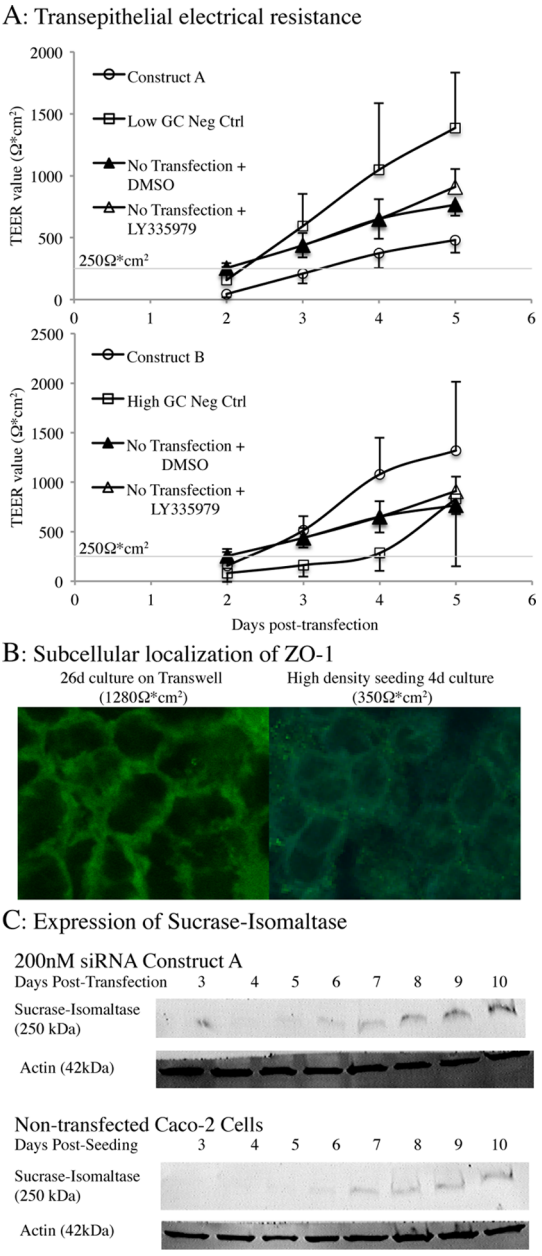


Figure 5. Characterization of the Caco-2 cell monolayers. (A) Effect of culture time and treatment on transepithelial electrical resistance, mean ± SD. (B) Representative immunofluorescence images demonstrating the subcellular localization of the tight junction protein ZO-1 at the cell−cell interfaces from untreated Caco-2 cells grown using traditional techniques for 26 days on semipermeable polycarbonate filters and from the treatment group with the lowest TEER value obtained at day 4 post-transfection. (C) Detection of sucrase-isomaltase by Western blot as a marker for the formation of a brush border membrane in Caco-2 cells grown on semipermeable polycarbonate filters.

For the experimental model to be valid, the transfected Caco-2 cells must form confluent monolayers and differentiate into enterocyte-like cells while *ABCB1* remains silenced. The cells formed tight junctions within 4 days of seeding the transfectants on semipermeable polycarbonate membranes, determined both by TEER values[37] >250 Ω·cm^2 and by immunofluorescence microscopy for ZO-1 subcellular localization as shown in Figure 5. Differentiation of Caco-2 cells was demonstrated by detection of the brush border enzyme sucrase-isomaltase by Western blot within five days of plating the transfectants with levels increasing until day 10 of culture on the Transwells (Figure 5).

To quantify the effects of siRNA-mediated silencing on Pgp function, we conducted both accumulation and transport studies of Rh123, a well-established Pgp substrate. In Figure 6, we demonstrated increased intracellular retention of Rh123 relative to untreated controls in Caco-2 monolayers grown on

dx.doi.org/10.1021/mp300668e | *Mol. Pharmaceutics* 2013, 10, 2323−2330

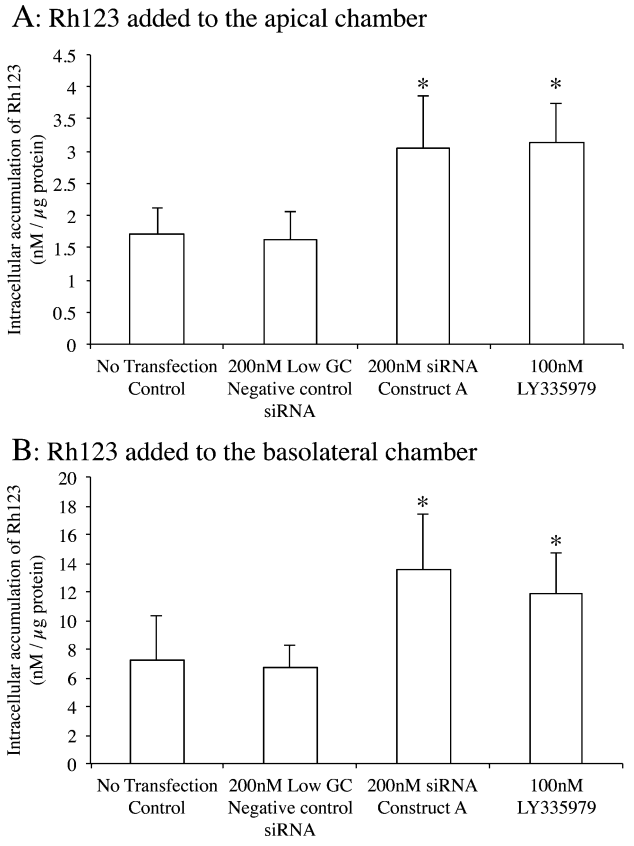


Figure 6. Effect of siRNA on the intracellular accumulation of rhodamine 123 in Caco-2 cells grown on semipermeable polycarbonate membranes. (A) Accumulation after rhodamine 123 was incubated on the apical face of the Caco-2 monolayer. (B) Accumulation after rhodamine 123 was incubated in the basolateral chamber of the Transwell. *p < 0.05 vs untransfected cells, n = 6 separate experiments.

polycarbonate membranes after transfection with a single dose of 200 nM siRNA (construct A) or after treatment with 100 nM LY335979. This observation was consistent when the Rh123 was added to the apical or to the basolateral chambers. To ensure reproducibility of these data, the experiment was repeated on six different passages of Caco-2 cells. Pgp function was further assessed by quantifying the transcellular flux of Rh123 both in the apical to basolateral direction and the basolateral to apical direction on Caco-2 cells in three separate experiments using cells at different passage numbers. The data shown in Figure 7 demonstrate that the siRNA-treated cells had statistically significant reductions in the ratio of the apparent permeability coefficients (efflux basolateral→apical relative to uptake apical→basolateral), consistent with treatment with the well-established Pgp inhibitor, LY335979.

DISCUSSION

Caco-2 cells are a well-established model for enterocytes, the absorptive cells of the digestive tract. These cells are often used in early screens to determine whether drug candidates are substrates for the efflux transporter Pgp. Current approaches utilizing small molecule inhibitors of Pgp are limited because of the nonspecific nature of the molecules.[14] In this paper, we tested the ability of chemically modified siRNA to target and suppress *ABCB1* in Caco-2 cells as potential specific inhibitors of Pgp function. We demonstrated a dose-dependent reduction

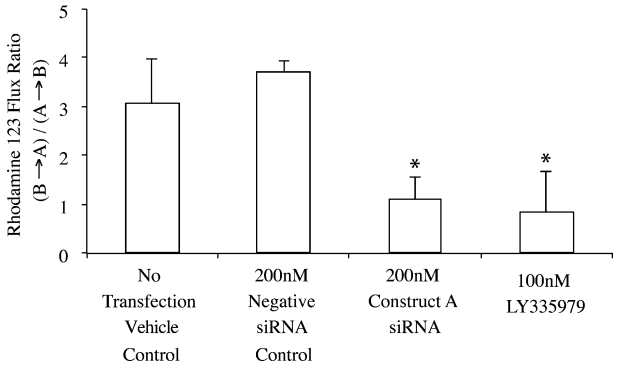


Figure 7. Effect of siRNA on the transcellular flux of rhodamine 123 across Caco-2 cells grown on semipermeable polycarbonate membranes. The apparent permeability coefficients for basolateral to apical (B→A) transport and for apical to basolateral (A→B) transport were determined under sink conditions for each group. The mean ratio of the two permeability coefficients is presented with the error bars representing one standard deviation. *p < 0.05 vs vehicle control cells, n = 3 separate experiments using different passage number Caco-2 cells.

in *ABCB1* mRNA accompanied by ablation of Pgp protein that lasted up to 10 days post-transfection. Silencing Pgp in this manner also decreased Pgp function, as shown by increased accumulation and reduced efflux transport of Rh123.

Under appropriate culture conditions, Caco-2 cells can spontaneously differentiate to a polarized monolayer with ordered microvilli and brush border enzymes on the apical face.[6,7,38,39] Unfortunately, while these characteristics make the cells a good model of enterocytes, they make effective transfections challenging.[25,26] Undifferentiated Caco-2 cells in suspension can be transfected, but under normal culture conditions the cells require 14−21 days from the time of seeding for full differentiation,[9] which exceeds the effective duration of silencing for transient transfection with siRNA in other cell lines with doubling times of 30 h,[40,41] comparable to Caco-2.[10,42] To overcome these hurdles, we transfected the cells while in suspension then seeded them at high density (3.75×10^5 cells/cm^2) onto polycarbonate membranes to reduce the time needed for differentiation, consistent with other efforts to reduce the culture time for Caco-2 cells.[10]

This is not the first demonstration of RNAi-mediated silencing of Pgp in Caco-2 cells. Several other groups have addressed the challenge of transfecting Caco-2 cells by creating Caco-2 cells with stable transfections of plasmids encoding shRNA sequences that are processed intracellularly to siRNA targeting *ABCB1* mRNA.[17,18,21] Cells are grown in the presence of a toxic aminoglycoside antibiotic in order to ensure that only cells retaining the plasmid survive due to the inclusion of a resistance gene encoded in the plasmid. This technique has several advantages including the suppression of Pgp over several passage numbers and the ability to select for cells that were successfully transfected. Unfortunately, there are also several limitations to this approach. The creation of a Caco-2 cell line encoding a plasmid that encodes the desired siRNA sequence requires molecular biology expertise. Treatment of some cell types with aminoglycosides is associated with changes to cell homeostasis, including calcium sensing[43] and the induction of the JNK stress pathway.[30,44] Caco-2 cells are a heterogeneous cell population, and by selecting for cells that are easily transfected a researcher may inadvertently select for a specific cell type rather than the mixed cell population that has

been characterized for drug transport.[5,8,9,11] Furthermore, it is difficult to titrate the dose of siRNA that the cells are exposed to by using the stable transfection approach; this can be associated with insufficient silencing or the induction of nonspecific effects.[23]

Our transient transfection approach addresses several of the limitations with stable transfections described above. The data shown in Figure 2 demonstrates a dose-dependent reduction in *ABCB1* mRNA at 5 days post-transfection for Caco-2 cells grown on polycarbonate membranes. By conducting dose−response studies, we are able to minimize the dose of siRNA the cells receive and reduce the probability of nonspecific effects.[23] We have not selected for positive transfectants but rather are demonstrating that our approach can decrease the expression of *ABCB1* in the entire Caco-2 cell population by >70%. Our use of commercially available chemically modified siRNA reduces the molecular biology expertise required to grow Caco-2 cells with decreased Pgp levels, and this technique should be easily reproducible by researchers with experience growing Caco-2 cells. No selection pressure is required to maintain Caco-2 down-regulation after a transient transfection, which minimizes the potential for off-target effects of aminoglycosides. Although transient transfections do not achieve the long-term suppression of target genes seen with stable transfections, we were able to demonstrate an absence of Pgp protein from day 4 until day 10 post-transfection (Figure 4) by using chemically modified siRNA. The time course suppression of Pgp levels using modified siRNA allowed Caco-2 cells grown at high density to form tight junctions and express the brush border hydrolase sucrase-isomaltase (Figure 5). We demonstrated the utility of this approach using a well-characterized substrate for Pgp, Rh123, as a model for drug uptake and transport using similar culture conditions to those reported for other siRNA-based transport experiments.[17,19,29] The intracellular accumulation data shown in Figure 6 and the transcellular flux data shown in Figure 7 suggest that reducing Pgp levels by siRNA increased Rh123 retention and reduced efflux transport to a similar extent as our positive control for Pgp inhibition, treatment with 100 nM LY335979.[45]

In this paper we provide evidence that transient transfection of Caco-2 cells using chemically modified siRNA can be used to selectively reduce Pgp levels associated with reduced functionality. This method has several advantages over traditional stable transfection techniques and can be easily scaled for high throughput screens. A recent publication demonstrated the use of siRNA to distinguish the contributions of Pgp and the breast cancer resistance protein to active transport of Imatinib.[19] Our technique could similarly be expanded to investigate the contributions of multiple transporters by cotransfecting cells with noninteracting siRNA sequences that suppress the expression of genes encoding membrane drug transporters. The simplicity of using transient transfections will reduce the time required to establish the contributions of each transporter by eliminating the need to isolate Caco-2 clones that were stably transfected with multiple plasmids prior to testing for the suppression of the target genes. The transfection protocol described in this manuscript requires no molecular biology expertise and will give researchers who have experience with Caco-2 drug transport studies a precise genetic tool to evaluate the contributions of Pgp and other transporters to the transcellular flux of medications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of the siRNA sequences, Western blots, and immunofluorescence microscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 604-822-4889. Fax: 604-822-3035. E-mail: Kishor.Wasan@ubc.ca.

Author Contributions

S.D.L. and J.O.-T. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Funding for this project was provided in the form of an operating grant to K.M.W. from the Canadian Institutes of Health Research. S.D.L. was supported by a Banting and Best Canada Graduate Scholarship Doctoral Award from the Canadian Institutes of Health Research and a Senior Graduate Trainee award from the Michael Smith Foundation for Health Research.

■ ABBREVIATIONS USED

Pgp, P-glycoprotein; siRNA, small interfering ribonucleic acid; RNAi, ribonucleic acid interference; RT-qPCR, reverse transcription−quantitative real time polymerase chain reaction; TEER, transepithelial electrical resistance; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; Rh123, rhodamine 123; RIPA, radioimmunoprecipitation assay

■ REFERENCES

(1) Sugano, K.; Kansy, M.; Artursson, P.; Avdeef, A.; Bendels, S.; Di, L.; Ecker, G. F.; Faller, B.; Fischer, H.; Gerebtzoff, G.; Lennernaes, H.; Senner, F. Coexistence of passive and carrier-mediated processes in drug transport. *Nature Rev. Drug. Discovery* **2010**, *9*, 597−614.

(2) Greiner, B.; Eichelbaum, M.; Fritz, P.; Kreichgauer, H. P.; von Richter, O.; Zundler, J.; Kroemer, H. K. The role of intestinal P-glycoprotein in the interaction of digoxin and rifampin. *J. Clin. Invest.* **1999**, *104*, 147−153.

(3) Wu, C.-Y.; Benet, L. Z. Predicting drug disposition via application of BCS: transport/absorption/elimination interplay and development of a biopharmaceutics drug disposition classification system. *Pharm. Res.* **2005**, *22*, 11−23.

(4) Amidon, G. L.; Lennernäs, H.; Shah, V. P.; Crison, J. R. A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharm. Res.* **1995**, *12*, 413−420.

(5) Hidalgo, I. J.; Raub, T. J.; Borchardt, R. T. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **1989**, *96*, 736−749.

(6) Pinto, M.; Robine-Leon, S.; Appay, M.; Kedubger, M.; Triadou, N.; Dussaulx, E.; Lacroix, B.; Simon-Assmann, P.; Haffen, K.; Fogh, J.; Zweibaum, A. Enterocyte-Like Differentiation and Polarization of the Human Colon Carcinoma Cell-Line Caco-2 in Culture. *Biol. Cell* **1983**, *47*, 323−330.

(7) Hauri, H. P.; Sterchi, E. E.; Bienz, D.; Fransen, J. A.; Marxer, A. Expression and intracellular transport of microvillus membrane hydrolases in human intestinal epithelial cells. *J. Cell Biol.* **1985**, *101*, 838−851.

(8) Englund, G.; Rorsman, F.; Rönnblom, A.; Karlbom, U.; Lazorova, L.; Gråsjö, J.; Kindmark, A.; Artursson, P. Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. *Eur. J. Pharm. Sci.* **2006**, *29*, 269−277.
(9) Hubatsch, I.; Ragnarsson, E. G. E.; Artursson, P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nature Protoc.* **2007**, *2*, 2111−2119.
(10) Sambuy, Y.; De Angelis, I.; Ranaldi, G.; Scarino, M. L.; Stammati, A.; Zucco, F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol. Toxicol.* **2005**, *21*, 1−26.
(11) Tavelin, S.; Gråsjö, J.; Taipalensuu, J.; Ocklind, G.; Artursson, P. Applications of epithelial cell culture in studies of drug transport. *Methods Mol. Biol.* **2002**, *188*, 233−272.
(12) Gottesman, M. M.; Ling, V. The molecular basis of multidrug resistance in cancer: the early years of P-glycoprotein research. *FEBS Lett.* **2006**, *580*, 998−1009.
(13) Aller, S. G.; Yu, J.; Ward, A.; Weng, Y.; Chittaboina, S.; Zhuo, R.; Harrell, P. M.; Trinh, Y. T.; Zhang, Q.; Urbatsch, I. L.; Chang, G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **2009**, *323*, 1718−1722.
(14) Szakács, G.; Paterson, J. K.; Ludwig, J. A.; Booth-Genthe, C.; Gottesman, M. M. Targeting multidrug resistance in cancer. *Nature Rev. Drug. Discovery* **2006**, *5*, 219−234.
(15) Krishna, R.; Mayer, L. D. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *Eur. J. Pharm. Sci.* **2000**, *11*, 265−283.
(16) Field, F. J.; Born, E.; Chen, H.; Murthy, S.; Mathur, S. N. Esterification of plasma membrane cholesterol and triacylglycerol-rich lipoprotein secretion in CaCo-2 cells: possible role of p-glycoprotein. *J. Lipid Res.* **1995**, *36*, 1533−1543.
(17) Watanabe, T.; Onuki, R.; Yamashita, S.; Taira, K.; Sugiyama, Y. Construction of a functional transporter analysis system using MDR1 knockdown Caco-2 cells. *Pharm. Res.* **2005**, *22*, 1287−1293.
(18) Celius, T.; Garberg, P.; Lundgren, B. Stable suppression of MDR1 gene expression and function by RNAi in Caco-2 cells. *Biochem. Biophys. Res. Commun.* **2004**, *324*, 365−371.
(19) Graber-Maier, A.; Gutmann, H.; Drewe, J. A New Intestinal Cell Culture Model To Discriminate the Relative Contribution of P-gp and BCRP on Transport of Substrates Such as Imatinib. *Mol. Pharmaceutics* **2010**716181628.
(20) Klein, S.; Zolk, O.; Fromm, M. F.; Schrödl, F.; Neuhuber, W.; Kryschi, C. Functionalized silicon quantum dots tailored for targeted siRNA delivery. *Biochem. Biophys. Res. Commun.* **2009**, *387*, 164−168.
(21) Li, J.; Volpe, D. A.; Wang, Y.; Zhang, W.; Bode, C.; Owen, A.; Hidalgo, I. J. Use of transporter knockdown Caco-2 cells to investigate the in vitro efflux of statin drugs. *Drug Metab. Dispos.* **2011**, *39*, 1196−1202.
(22) Mello, C. C.; Conte, D. Revealing the world of RNA interference. *Nature* **2004**, *431*, 338−342.
(23) Hannon, G. J.; Rossi, J. J. Unlocking the potential of the human genome with RNA interference. *Nature* **2004**, *431*, 371−378.
(24) Wu, H.; Hait, W. N.; Yang, J.-M. Small interfering RNA-induced suppression of MDR1 (P-glycoprotein) restores sensitivity to multidrug-resistant cancer cells. *Cancer Res.* **2003**, *63*, 1515−1519.
(25) Kovbasnjuk, O.; Mourtazina, R.; Baibakov, B.; Wang, T.; Elowsky, C.; Choti, M. A.; Kane, A.; Donowitz, M. The glycosphingolipid globotriaosylceramide in the metastatic transformation of colon cancer. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 19087−19092.
(26) Laprise, P.; Viel, A.; Rivard, N. Human homolog of disc-large is required for adherens junction assembly and differentiation of human intestinal epithelial cells. *J. Biol. Chem.* **2004**, *279*, 10157−10166.
(27) Ghartey-Tagoe, E. B.; Babbin, B. A.; Nusrat, A.; Neish, A. S.; Prausnitz, M. R. Plasmid DNA and siRNA transfection of intestinal epithelial monolayers by electroporation. *Int. J. Pharm.* **2006**, *315*, 122−133.
(28) Chang, K.; Marran, K.; Valentine, A.; Hannon, G. J. RNAi in cultured mammalian cells using synthetic siRNAs. *Cold Spring Harbor Protoc.* **2012**, *2012*, 957−961.
(29) Shirasaka, Y.; Sakane, T.; Yamashita, S. Effect of P-glycoprotein expression levels on the concentration-dependent permeability of drugs to the cell membrane. *J. Pharm. Sci.* **2008**, *97*, 553−565.
(30) Sugahara, K.; Rubel, E. W.; Cunningham, L. L. JNK signaling in neomycin-induced vestibular hair cell death. *Hear. Res.* **2006**, *221*, 128−135.
(31) Lee, S. H.; Sinko, P. J. siRNA—getting the message out. *Eur. J. Pharm. Sci.* **2006**, *27*, 401−410.
(32) Clayburgh, D. R.; Rosen, S.; Witkowski, E. D.; Wang, F.; Blair, S.; Dudek, S.; Garcia, J. G. N.; Alverdy, J. C.; Turner, J. R. A differentiation-dependent splice variant of myosin light chain kinase, MLCK1, regulates epithelial tight junction permeability. *J. Biol. Chem.* **2004**, *279*, 55506−55513.
(33) Niwa, R.; Slack, F. J. Ins and Outs of RNA Interference Analysis. In *Evaluating Techniques in Biomedical Research* [Online]; Cell Press: Cambridge, MA, 2007; pp 34−36. http://www.cell.com/cellpress/ETBR (accessed Nov 1, 2012).
(34) Muller, C.; Laurent, G.; Ling, V. P-Glycoprotein stability is affected by serum deprivation and high cell density in multidrug-resistant cells. *J. Cell. Physiol.* **1995**, *163*, 538−544.
(35) Zhang, W.; Ling, V. Cell-cycle-dependent turnover of P-glycoprotein in multidrug-resistant cells. *J. Cell. Physiol.* **2000**, *184*, 17−26.
(36) Xie, Y.; Burcu, M.; Linn, D. E.; Qiu, Y.; Baer, M. R. Pim-1 kinase protects P-glycoprotein from degradation and enables its glycosylation and cell surface expression. *Mol. Pharmacol.* **2010**, *78*, 310−318.
(37) Buzza, M. S.; Netzel-Arnett, S.; Shea-Donohue, T.; Zhao, A.; Lin, C.-Y.; List, K.; Szabo, R.; Fasano, A.; Bugge, T. H.; Antalis, T. M. Membrane-anchored serine protease matriptase regulates epithelial barrier formation and permeability in the intestine. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 4200−4205.
(38) Rousset, M. The human colon carcinoma cell lines HT-29 and Caco-2: two in vitro models for the study of intestinal differentiation. *Biochimie* **1986**, *68*, 1035−1040.
(39) Lisanti, M. P.; Caras, I. W.; Davitz, M. A.; Rodriguez-Boulan, E. A glycophospholipid membrane anchor acts as an apical targeting signal in polarized epithelial cells. *J. Cell Biol.* **1989**, *109*, 2145−2156.
(40) Bartlett, D. W.; Davis, M. E. Effect of siRNA nuclease stability on the in vitro and in vivo kinetics of siRNA-mediated gene silencing. *Biotechnol. Bioeng.* **2007**, *97*, 909−921.
(41) Bartlett, D. W.; Davis, M. E. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res.* **2006**, *34*, 322−333.
(42) Grès, M. C.; Julian, B.; Bourrié, M.; Meunier, V.; Roques, C.; Berger, M.; Boulenc, X.; Berger, Y.; Fabre, G. Correlation between oral drug absorption in humans, and apparent drug permeability in TC-7 cells, a human epithelial intestinal cell line: comparison with the parental Caco-2 cell line. *Pharm. Res.* **1998**, *15*, 726−733.
(43) Brown, E. M.; Gamba, G.; Riccardi, D.; Lombardi, M.; Butters, R.; Kifor, O.; Sun, A.; Hediger, M. A.; Lytton, J.; Hebert, S. C. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature* **1993**, *366*, 575−580.
(44) Pirvola, U.; Xing-Qun, L.; Virkkala, J.; Saarma, M.; Murakata, C.; Camoratto, A. M.; Walton, K. M.; Ylikoski, J. Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. *J. Neurosci.* **2000**, *20*, 43−50.
(45) Dantzig, A. H.; Shepard, R. L.; Cao, J.; Law, K. L.; Ehlhardt, W. J.; Baughman, T. M.; Bumol, T. F.; Starling, J. J. Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. *Cancer Res.* **1996**, *56*, 4171−4179.