Silencing of breast cancer resistance protein expression by lentiviral shRNA transduction particles in Caco-2 cells

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ABSTRACT

Caco-2 is a human intestinal cell line that expresses three drug efflux transporters in the apical plasma membrane: breast cancer resistance protein (BCRP, ABCG2), P-glycoprotein (P-gp; ABCB1) and MRP-2 (ABCC2). BCRP and P-gp, in particular, are believed to play a significant role in pharmacokinetics due to their expression at several key interfaces in the body that determine the ADME properties of drugs. In addition, over-expression of P-gp or BCRP in cancer cells may serve as a mechanism of tumor resistance to cancer chemotherapeutic drugs. The goal of this project was to engineer and characterize Caco-2 cell clones with stable knockdown (KD) of BCRP expression. The results of qPCR showed that expression of BCRP mRNA was knocked down to different degrees in each of the five lines that were characterized, with a minimum of 97% silencing in Clone D. Silencing of BCRP gene expression is maintained for at least 25 passages. By Western blot, expression of BCRP protein was also reduced significantly. Functionally, BCRP knockdown is reflected in a significant reduction of the efflux ratio (aparent permeability [P_app] in the basolateral to apical [B-A] direction divided by the A-B P_app) of E3S, a substrate of BCRP. In conclusion, stable BCRP knockdown cell lines were produced by transduction of Caco-2 cells with lentiviral shRNA. These knockdown cells constitute a novel tool for studying BCRP-mediated drug resistance and interaction of BCRP with other ABC transporters.

INTRODUCTION

BCRP is a member of the ATP-binding cassette (ABC) transporter G family and is also known as ABCG2. Over-expression of BCRP is associated with high levels of resistance to a variety of anticancer agents by enhancing their efflux (Maiz QC, 2005). A potential approach to overcoming drug resistance is the use of potent and specific inhibitors of BCRP transport. However, clinical success using inhibitors to reverse BCRP-mediated resistance has been modest due in part to toxicity problems and pharmacokinetic interactions (Fojo T et al., 2003). Recently, the expression of BCRP in Caco-2 cells was shown to be reduced using RNAi technology (Priebsch et al., 2006, Li WT et al., 2006 and Ee PL et al., 2004). However, RNAi synthesized in vitro reduces gene expression only transiently and expression is restored a few days after transfection. It is also often limited to cells that are easily transfected. In addition, very little is known about the stability of inhibition of gene expression after several cell passages. Here, our hypothesis is that lentiviral particles encoding a short hairpin shRNA could produce long-lasting silencing of BCRP expression in Caco-2 cells. The results showed that stable, sequence-specific down-regulation of human BCRP gene expression and BCRP translation can be reduced by endogenous expression of shRNA carried by lentiviral vectors in Caco-2 cells.

MATERIALS AND METHODS

shRNA Library of BCRP: Viral vector-based shRNA lentiviral transduction particles of human BCRP (GenBank Accession No. NM 004827) were used to infect and integrate five BCRP shRNA constructs into Caco-2 cells following the manufacturer’s protocol (Sigma, St. Louis, MO).

Cell Culture and Establishment of Stable shRNA/BCRP Caco-2 cells: The parental cell line, C2BBe1, was derived from the Caco-2 cell line (ATCC Accession Number CRL-2102). Cells were cultured in DMEM containing 10% FBS. After Caco-2 cells were transduced with shRNA as described above, they were cultured in the same medium containing puromycin as the selection marker.

Western Blot: Cell protein extracts were prepared in RIPA lysis buffer (Santa Cruz Biotechnologies, CA). Forty-five (45) µg of protein were resolved on 10% SDS-polyacrylamide gels. After transfer to the blotting membrane, immunoblot analysis with monoclonal antibody (BXP-21) from Sigma (St Louis, MO), was used to probe for human BCRP. To assure equivalent protein loading, the membrane was simultaneously incubated with an antibody for β-actin. Protein-antibody complexes were detected by chemiluminescence (Pierce, Rockford, IL).

RT-PCR and qPCR: RT-PCR was performed using the SuperScript III One Step RT-PCR System (Invitrogen, Carlsbad, CA). Sequences for BCRP and β-actin (for normalization) primers were designed using primer design software for PrimerExpress (Applied Biosystems) and Primer3 (Whitehead Institute for Biomedical Research). For quantitative mRNA expression analysis, the cDNA synthesis reaction was performed using Postscript First Strand CDNA Synthesis Kit (New England Biolabs, Cat# E6500L, Ipswitch, MA). Real-time PCR (qPCR) protocols were applied using LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany). All the sequences of primers and probes of qPCR were designed using Universal Probe Library (UPL, Roche, Basel, Switzerland).

Immunofluorescence microscopy: Cells were cultured on poly-D-lysine pre-coated cover slips (BD Biosciences, Bedford, MA) and grown to confluence. The expression of cell monolayers. The AAPS Journal 2005; 7(1): E118-133.

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Permeability Studies: The BCRP knockdown and control cells were grown on Transwell inserts to a monolayer density of 100,000 cells/cm2. After 24 hours, transcellular electrical resistance (TEER) was measured, and bidirectional transport experiments were conducted using a solution containing 10 µM propranolol, 10 µM atenolol and 5 mM estrone-3-sulfate (E3S) for 2 hours. All samples were analyzed by LC/MS/MS.

Calculation: The apparent permeability coefficient, Papp, was calculated using the following equation: Papp=(ΔC/Δt)/(V x A x Co), where ΔC/Δt is the slope of the cumulative concentration in the receiver compartment versus time in µM, V is the volume of the receiver compartment in cm3, A is the area of the cell monolayer, and Co is the initial concentration of the dosing solution.

RESULTS

• Reduction of BCRP gene expression by lentiviral shRNA: Results of RT-PCR, qPCR and Western Blot (Figure 1, 2 and 3) showed that expression of human BCRP mRNA and protein in all five shRNA/BCRP KD cells was reduced with a maximum of 97% silencing in Clone D. Immunofluorescence (Figure 6) showed that gene expression was a strong overall inhibition in the BCRP signal in knockdown cells compared to vector control cells.

• Stability of gene knockdown: Results (Figure 4) show that silencing of BCRP gene expression lasted for at least 25 passages, which is much longer than with transient transfections of RNAi (Priebsch et al., 2008, Li WT et al., 2006).

• Reduction of BCRP-mediated efflux: All five shRNA/BCRP clone cells showed a significant reduction in the efflux ratio (PappB/A→PappB/A) of E3S (ranged from 2.60 to 14.42) compared to vector control cells (25.58, see Table 1). The transcellular transport of the reference compounds, atenolol and propranolol, was not altered by BCRP knockdown.

CONCLUSION

• Expression of the BCRP gene is effectively silenced by transduction of Caco-2 cells with lentiviral shRNA. Our results support a recent study in that it demonstrated the effectiveness of shRNA knockdown in mammalian cells (Moffat et al., 2006).

• The inhibition of the BCRP gene expression persisted over a wide range of cell passages.

• E3S efflux was significantly decreased in the shRNA/BCRP clone cells. This result further confirms that shRNA/BCRP is an effective approach to modulate BCRP-mediated transport function of cell monolayers.

• In conclusion, a series of stable BCRP knockdown cell lines were produced by transduction of Caco-2 cells with lentiviral shRNA. One clone in particular should be a useful model for identifying and characterizing P-gp substrates and inhibitors without appreciable interference from BCRP. In addition, it can be useful in conjunction with wild-type or vector control Caco-2 cells to identify BCRP substrates. These transporter-specific knockdown cells, which are a novel and effective laboratory tool to evaluate the role of BCRP in drug and nutrient efflux, constitute a major improvement over the relatively non-specific pharmacologic inhibitors currently used to identify substrates and inhibitors of efflux transporters.

REFERENCES


