**ABSTRACT**

Purpose. To characterize the transport functions of a Caco-2 subclone in which BCRP transporter was down-regulated by the RNA interference (RNAi) technique. Methods. Caco-2 cells were transduced with lentivirus containing human BCRP-targeting or non-targeting shRNAs to create a BCRP knockdown clone (CPT-B1) and a vector control clone (VC). CPT-B1 and VC cells were grown on collagen-coated microporous polycarbonate membranes in Transwell plates. The barrier properties of the monolayers were assessed by measuring their electrical resistance and the transport of the passive diffusion markers atenolol and propranolol. Functional activities of P-gp and BCRP in these engineered cells were evaluated, respectively, by the bi-directional transport of digoxin and extreme-3-sulfate (E3S). Sample analyses were conducted with LC/MS/MS. Results. CPT-B1 and VC cell monolayers matured approximately 15-30 days post-seeding. Both cell types exhibited similar barrier properties with TEER > 300 Ω·cm² and distinct separation between low permeability substrates (Papp < 1x10⁻⁶ cm/s) and the high permeability propranolol (Papp > 1x10⁻⁴ cm/s). BCRP activity in CPT-B1 cells was significantly reduced (E3S efflux ratio < 4) compared to VC cells (E3S ratio > 20), whereas P-gp activity was preserved in both cells (digoxin efflux ratio > 10).

Conclusion. CPT-B1 cells exhibited similar barrier properties to those present in vector control Caco-2 cells. This newly generated cell model can serve as an in vitro tool for studying and distinguishing drug transport processes involving Pgp and/or BCRP.

**INTRODUCTION**

Breast cancer resistance protein (BCRP) encoded by ABCG2 gene is an ABC efflux transporter that transports sulfconjugated organic anions as well as hydrophobic and amphiphilic compounds. It has been reported that BCRP restricts the intestinal absorption and fetal penetration of its substrates. BCRP has also been found at the luminal side of human and porcine brain capillary endothelial cells, which suggests that BCRP may serve as an efflux transporter to restrict brain penetration of drugs together with Pgp. However, in vivo results with knockout mice have been controversial so far. Therefore, the physiological functions of BCRP at the luminal membrane of brain capillaries and other organs need to be further characterized.

The human colon adenocarcinoma cell line, Caco-2, expresses numerous transport systems, including the efflux transporters, P-glycoprotein (Pgp), breast cancer resistance protein (BCRP), and multidrug resistance transporters (MRPs). Owing to its similar characteristics to the small intestine and colon, the Caco-2 cell model has been widely used to study various intestinal transport processes. Yet, expression of multiple transporters often complicates interpretation of results. The involvement of specific transport pathway(s) cannot be readily estimated. For this reason, Absorption Systems has adopted the strategy of knocking down specific transporter genes in Caco-2 cells using the RNA interference (RNAi) technique. This approach selectively downregulates transporter genes, thus providing opportunities to study specific transport processes in cells of human origin while other transporters, such as apical or basolateral uptake transporters, remain intact. The overall goal of this project is to create a panel of transporter knockdown Caco-2 cells to enable the study of the roles of efflux transporters, P-glp (Pgp), breast cancer resistance protein (BCRP), and MRPs, in drug transport. BCRP knockdown Caco-2 clone (CPT-B1) and vector control clone (VC) were generated by transducing Caco-2 cells with lentivirus containing human BCRP-targeting and non-targeting shRNAs to create a BCRP knockdown clone (CPT-B1) and vector control clone (VC). The overall goal of this project is to create a panel of transporter knockdown Caco-2 cells to enable the study of the roles of efflux transporters, P-glp (Pgp), breast cancer resistance protein (BCRP), and MRPs, in drug transport.

**METHODS**

Establishment of stable BCRP knockdown Caco-2 cells: Lentiviral particles containing human BCRP-targeting and non-targeting shRNAs were used to transduce Caco-2 cells following the manufacturer’s protocol (Sigma-Aldrich, MO). Stably transduced cells were cultured in DMEM containing 10% FBS with puromycin as the selecting agent.

**Real-time PCR:** cDNA synthesis reaction was performed using Postscript First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). Real-time PCR protocol was applied using BCRP-specific primers and probe with Light Cycle 480 instrument (Roche Diagnostics, Mannheim, Germany). All the sequences of primers and probes of qPCR were validated using the NCBI database. PCR efficiency was estimated at <7% of control.

**Functional characterization of RNAi**

**Barrier properties and transport functions of VC and CPT-B1, assayed on days 15, 20, 25, and 30 post-seeding, are summarized in Table 1 and graphically illustrated in Figure 3. The average TEER values of CPT-B1 cells between 15 and 30 days were 600 - 1000 Ω·cm², indicating that BCRP cells formed a monolayer with acceptable barrier properties by day 15. Both CPT-B1 and VC cells showed distinct separations in the transport rates of the low- and high-permeability diffusion markers atenolol and propranolol. Atenolol Papp in CPT-B1 cells was 1.9 x 10⁻⁶ cm/s on day 15 then decreased below 0.7 x 10⁻⁶ cm/s, suggesting an optimal range for transport experiments between days 20 and 30. Digoxin, a Pgp substrate, was actively effluxed across both VC and CPT-B1 cell monolayers, indicating active Pgp function. Between day 20 and 30, the digoxin efflux ratio was >10 in CPT-B1 cells. E3S, a BCRP substrate, was actively effluxed across VC cell monolayers with efflux ratio >30, whereas E3S efflux was significantly reduced across CPT-B1 cell monolayers. Using VC cells as reference, residual BCRP activity in CPT-B1 cells was estimated at <7% of control.

**RESULTS**

**Relative mRNA levels by real-time, quantitative PCR are listed in Table 1. Compared to wild-type Caco-2 cells, BCRP levels in VC cells remained virtually unchanged, whereas it decreased to 3% in CPT-B1 cells. Other efflux transporters, MRP-2 and Pgp, remained relatively unchanged in VC and CPT-B1 cells.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>VC mRNA expression</th>
<th>CPT-B1 mRNA expression</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCRP</td>
<td>1.00</td>
<td>0.03 ± 0.003</td>
<td>0.03</td>
</tr>
<tr>
<td>MRP-2</td>
<td>1.00</td>
<td>0.71 ± 0.02</td>
<td>1.21 ± 0.27</td>
</tr>
<tr>
<td>Pgp</td>
<td>1.00</td>
<td>0.77 ± 0.029</td>
<td>0.80 ± 0.16</td>
</tr>
</tbody>
</table>

**Table 1. Relative mRNA Levels of Efflux Transporters**

**Conclusion**

Molecular evidence indicated a substantial reduction in BCRP mRNA and protein levels in CPT-B1 cells. The cell line exhibited similar barrier properties to vector control Caco-2 cells. BCRP activity was greatly reduced, while Pgp activity was preserved, in CPT-B1 cells. This newly generated cell model represents a valuable in vitro tool for studying and distinguishing drug transport processes involving Pgp and/or BCRP.