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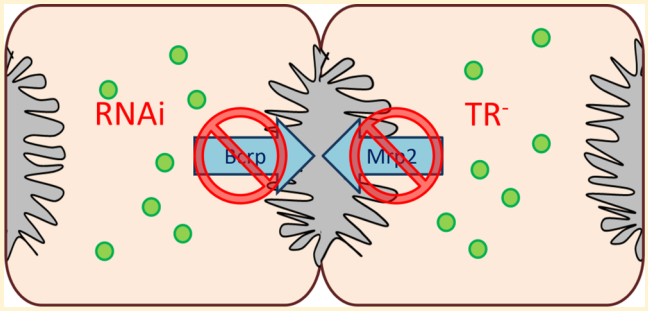
# An Experimental Approach To Evaluate the Impact of Impaired Transport Function on Hepatobiliary Drug Disposition Using Mrp2-Deficient TR⁻ Rat Sandwich-Cultured Hepatocytes in Combination with Bcrp Knockdown

Kyunghee Yang,[†,‡] Nathan D. Pfeifer,[†,‡] Rhiannon N. Hardwick,[§] Wei Yue,[†,∥] Paul W. Stewart,[⊥] and Kim L. R. Brouwer*[,†,§,#]

[†]Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, [§]Curriculum in Toxicology, and [⊥]Department of Biostatistics, UNC Gillings School of Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, 27599-7569, United States

Supporting Information

**ABSTRACT:** Breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 2 (MRP2) are members of the ATP binding cassette (ABC) transporter family located in the canalicular membrane of hepatocytes that mediate biliary excretion of many drugs and endogenous compounds. BCRP and MRP2 have overlapping substrate profiles. Predicting drug disposition in the setting of altered transport function has important clinical significance. This investigation was designed to establish an *in vitro* model system to evaluate the impact of impaired Mrp2 and Bcrp function on hepatobiliary drug disposition. To achieve Bcrp knockdown by RNA interference (RNAi), sandwich-cultured hepatocytes (SCH) from Mrp2-deficient (TR⁻) and wild-type (WT) rats were infected with adenoviral vectors to express shRNA targeting Bcrp (Ad-siBcrp) at multiplicity of infection (MOI) of 1−10. MOI of 5 was identified as optimal. At MOI of 5, viral infection as well as WT or TR⁻ status was statistically significant predictors of the rosuvastatin (RSV) biliary excretion index (BEI), consistent with the known role of Bcrp and Mrp2 in the biliary excretion of RSV *in vivo* in rats. Relative to WT rat SCH, marginal mean BEI (%) of RSV in TR⁻ rat SCH decreased by 28.6 (95% CI: 5.8−51.3). Ad-siBcrp decreased marginal mean BEI (%) of RSV by 13.3 (7.5−9.1) relative to SCH infected with adenoviral vectors expressing a nontargeting shRNA (Ad-siNT). The BEI of RSV was almost ablated in TR⁻ rat SCH with Bcrp knockdown (5.9 ± 3.0%) compared to Ad-siNT-infected WT rat SCH (45.4 ± 6.6%). These results demonstrated the feasibility of Bcrp knockdown in TR⁻ rat SCH as an *in vitro* system to assess the impact of impaired Bcrp and Mrp2 function. At MOI of 5, viral infection had minimal effects on RSV total accumulation, but significantly decreased marginal mean taurocholate total accumulation (pmol/mg of protein) and BEI (%) by 9.9 (7.0−12.8) and 7.5 (3.7−11.3), respectively, relative to noninfected SCH. These findings may be due to off-target effects on hepatic bile acid transporters, even though no changes in protein expression levels of the hepatic bile acid transporters were observed. This study established a strategy for optimization of the knockdown system, and demonstrated the potential use of RNAi in SCH as an *in vitro* tool to predict altered hepatobiliary drug disposition when canalicular transporters are impaired.




**KEYWORDS:** *breast cancer resistance protein (BCRP/Bcrp), multidrug resistance-associated protein 2 (MRP2/Mrp2), RNA interference (RNAi), sandwich-cultured hepatocytes (SCH)*

## INTRODUCTION

Hepatocytes are polarized cells with distinct apical and basolateral domains. Transport proteins on the apical membrane are responsible for excretion of compounds into the bile canaliculus, whereas basolateral transport proteins mediate influx into hepatocytes and efflux back to sinusoidal blood. Hepatic canalicular and basolateral transport proteins play important roles in regulating the pharmacologic and toxicologic effects of many drugs by mediating hepatocellular exposure. Breast cancer resistance protein (BCRP), a member of the ATP-binding cassette (ABC) transporter family, is a half-transport protein that forms a functional homodimer or oligomer.[1,2] BCRP is highly expressed in the canalicular membrane of hepatocytes as well as in the intestine, breast, and placenta.[3] BCRP substrates include glucuronide and sulfate conjugates [e.g., estrone-sulfate,

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estradiol-17$\beta$-D-glucuronide ($E_2$17G), SN-38 glucuronide], anticancer drugs (e.g., irinotecan, SN-38, methotrexate, daunorubicin, doxorubicin), and some statins [e.g., pitavastatin, rosuvastatin (RSV)].[4−9] The pharmacokinetics and pharmacodynamics of these drugs may be affected by modulation of BCRP expression and/or function resulting from genetic polymorphisms or drug−drug interactions (DDIs).

Multidrug resistance-associated protein 2 (MRP2) is another member of the ABC transporter family that is expressed in the canalicular membrane of hepatocytes. Many drugs are substrates for both BCRP and MRP2 including RSV, methotrexate, doxorubicin, SN-38, and SN-38 glucuronide. Overlapping substrate specificity can make it challenging to predict the impact of altered function of one or more transport proteins on the hepatic and systemic exposure of substrates. A significant change in drug exposure is expected following loss-of-function of one or more transport pathways when clearance by that particular pathway (apical or basolateral) exceeds 50% of total clearance.[10] Moreover, altered drug exposure as a result of impaired transport function depends on the remaining excretion routes, such as complementary efflux transporters on the same membrane (e.g., canalicular excretion into bile) or alternative efflux transporters on the opposite membrane (e.g., basolateral excretion into sinusoidal blood). Thus, *in vitro* and *in vivo* models to assess changes in hepatocellular accumulation and routes of excretion of compounds in the setting of impaired transport function are greatly needed.

Several model systems have been proposed to assess the role of BCRP and MRP2 in the disposition of a substrate. One approach is the use of specific BCRP and MRP2 inhibitors in hepatocytes. However, inhibitors of BCRP (e.g., GF120918, Ko134, fumitremorgin C, mitoxantrone, novobiocin) and MRP2 (e.g., MK-571, benzbromarone) may not be specific enough to allow assessment of the role of individual proteins.[11−13] Similarly, specific substrates have been employed in hepatocytes and transport protein overexpressing cells to evaluate quantitatively the contribution of an individual hepatic uptake transporter [i.e., relative activity factor (RAF) method],[14] but "specific" BCRP and MRP2 substrates are lacking due to the aforementioned overlapping substrate spectrum of these transport proteins. Although the use of transient or stably transfected cell lines expressing one or more transport proteins is a popular approach to assess the role of individual proteins in substrate disposition, this approach may be misleading. Expression levels of transport proteins in these *in vitro* systems may not be representative of the true physiologic state, and metabolic systems as well as other regulatory factors impacting hepatobiliary disposition of substrates may be absent or present at low levels, depending on the *in vitro* system. Thus, transport of substrates by a specific protein in transporter-expressing cells *in vitro* does not guarantee that the transporter will play a key role in substrate disposition *in vivo*.

Another approach is the use of naturally occurring, genetically deficient rodents or genetically engineered animals lacking a specific transport protein. Mrp2-deficient Wistar ($TR^-$) rats and Eisai-hyperbilirubinemic Sprague−Dawley rats have been used to delineate the role of Mrp2 in drug disposition *in vivo*.[15,16] Likewise, Bcrp knockout (*Abcg2*−/−) mice have been used to investigate whether Bcrp is involved in the disposition of drugs such as RSV, methotrexate, mitoxantrone, and pitavastatin.[8,17−19] Recently, Mrp2 knockout rats and Bcrp knockout rats were generated using zinc finger nuclease technology, and knockout phenotypes in these rats were characterized using sulfasalazine and 5-(and 6)-carboxy-2′,7′-dicholorofluorescein as probes for Bcrp and Mrp2 function, respectively.[20,21] While *in vivo* pharmacokinetic studies in these models provide insight regarding overall drug distribution and excretion, sandwich-cultured hepatocytes (SCH) prepared from rodents lacking a specific transport protein allow assessment of altered hepatobiliary disposition in isolation from other organs.[22−24]

RNA interference (RNAi) is one approach to explore the consequences of impaired protein function, and has been used to knock down transport proteins in the SCH system. Tian et al. transfected rat SCH with synthetic small interfering RNA (siRNA) to specifically knock down protein levels of Mrp2 and Mrp3; approximately 50% knockdown was achieved using this approach.[25] Knockdown of mRNA and protein levels of OATP1B1, OATP1B3, and OATP2B1 using siRNA has been reported in human SCH.[26] In primary cells, it is technically challenging to reach high transfection efficiency. Delivery of short hairpin (sh) RNA using an adenoviral vector system resulted in high infection efficiency leading to high knockdown efficiency.[27] Rat SCH infected with adenoviral vectors expressing shRNA targeting Bcrp exhibited a significant decrease in protein expression and activity of this canalicular transport protein; the disposition of digoxin, a P-gp substrate, and the expression of some other transport proteins was not affected.[28]

To date, primary hepatocyte models lacking multiple transport proteins have not been established. Such a model may be of particular importance if it mimics the physiological condition when an administered drug inhibits the function of multiple transporters due to the nonspecific nature of transport inhibitors. The purpose of this investigation was to develop an *in vitro* model system to assess the consequences of altered transport function when multiple proteins are involved in hepatic excretion. Knockdown of Bcrp in SCH from $TR^-$ and wild-type (WT) rats was developed as an *in vitro* system to assess the impact of impaired function of Bcrp and/or Mrp2 using probe substrates. RSV, a Bcrp and Mrp2 substrate, and taurocholate (TC), a model bile acid that is not transported by Bcrp and Mrp2, were selected as probe substrates for investigation. This report describes a two-stage statistical analysis strategy for optimizing the knockdown system.

## EXPERIMENTAL METHODS

**Chemicals.** Penicillin−streptomycin solution, dexamethasone, Hanks' balanced salt solution (HBSS; with or without $Ca^{2+}$ and $Mg^{2+}$), collagenase (type IV), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and MEM nonessential amino acids were purchased from Invitrogen (Carlsbad, CA). Insulin/transferrin/selenium culture supplement, BioCoat culture plates, and Matrigel extracellular matrix were purchased from BD Biosciences Discovery Labware (Bedford, MA). [$^3$H]TC (5 Ci/mmol; purity >97%) and [$^3$H]$E_2$17G (50.3 Ci/mmol; purity >97%) were purchased from Perkin-Elmer (Waltham, MA). [$^3$H]RSV (10 Ci/mmol; purity >99%) was purchased from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

**Packaging of Recombinant shRNA-Expressing Adenoviral Vectors.** Adenoviral vectors expressing small hairpin RNA (shRNA) targeting rat Bcrp (Ad-siBcrp), rat Mrp2 (Ad-siMrp2) or a nontargeted control shRNA (Ad-siNT) were packaged as published previously using the Adeno-XTM ViralTrak DsRed-Express Promoterless Expression System 2 (Clontech Labo-

ratories, Mountain View, CA).[28] The titer of adenoviral vectors was measured using Adeno-X Rapid Titer Kit (Clontech Laboratories, Mountain View, CA). siRNA sequences targeting the rat Bcrp gene at positions 288−306 relative to the start codon were published previously;[28] siRNA sequences targeting rat MRP2 at positions 4257−4275 and a nontarget siRNA (Ad-siNT) control sequence (ATGTATTGGCCTGTATTAG) were obtained from Darmacon (Chicago, IL).

**Isolation and Culture of Rat SCH.** Primary rat hepatocytes were isolated from male Wistar (220−300 g, Charles River Laboratories, Inc., Wilmington, MA) and TR$^-$ (220−300 g, bred in-house; breeding stock obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) rats and seeded onto 24-well collagen-coated plates at a density of $0.35 \times 10^6$ cells/well in seeding medium (DMEM containing 5% fetal bovine serum, 10 $\mu$M insulin, 1 $\mu$M dexamethasone, 2 mM L-glutamine, 1% MEM nonessential amino acids, 100 units of penicillin G sodium, and 100 $\mu$g of streptomycin). One hour after seeding, hepatocytes were infected with Ad-siBcrp, Ad-siMrp2, or Ad-siNT at multiplicity of infection (MOI) of 1, 3, 5, and 10 by replacing the seeding medium with fresh seeding medium containing virus. On the next day, media including viruses was removed, and cells were overlaid with Matrigel at a concentration of 0.25 mg/mL in 0.5 mL/well ice-cold culture medium (DMEM supplemented with 0.1 $\mu$M dexamethasone, 2 mM L-glutamine, 1% MEM nonessential amino acids, 100 units of penicillin G sodium, 100 $\mu$g of streptomycin, and 1% insulin/transferrin/selenium). Culture medium was changed every 24 h until experiments were performed on day 4.

**Quantitative Real-Time Polymerase Chain Reaction (RT-PCR).** Total RNA was isolated from cell lysates using the ABI RNA isolation system (Applied Biosystems, Foster City, CA). mRNA levels of rat Bcrp and $\beta$-actin (internal control) were measured by TaqMan real-time RT-PCR using an ABI Prism 7700 System (Applied Biosystems) as described previously.[29] The TaqMan probe and primer sequences (5′−3′) used for rat Bcrp were as follows: forward (TGGATTGCCAGGCGTTCATT), reverse (GTCCCAGTATGACTGTAACAA), and probe (CTGCTCGGGAATCCTCAAGCTTCTG). Rat $\beta$-actin was detected using the following probe and primer sequences: forward (TGCCTGACGGTCAGGTCA), reverse (CAGGAAGGAAGGCTGGAAG), and probe (CACTAATCGGCAATGAGCGGTTCCG). Fold changes in mRNA levels of Bcrp were evaluated after normalizing the gene expression levels by those of $\beta$-actin ($2^{-\Delta\Delta Ct}$ method) as previously described.[30]

**Immunoblots.** Cells were washed with HBSS and lysis buffer containing 1% NP-40, 0.1% $Na^+$-deoxycholate, 1 mM EDTA, and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) was applied. Protein concentrations were measured by the BCA assay (Pierce, Rockford, IL). Whole-cell lysates (15 $\mu$g) were resolved on NuPAGE 4 to 20% Bis-Tris gel (Invitrogen, Carlsbad, CA), and the proteins were transferred to nitrocellulose membranes. After blocking in 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) for 30 min, blots were incubated overnight at 4 °C with the following antibodies: Bcrp (BXP-53), Mrp2 (M2III-6), Mrp4 (M4I-10), and P-gp (C219) (Alexis Biochemicals, San Diego, CA); Oatp1a1 (AB3570P, Millipore, Billerica, MA); Bsep (K44, kind gift from Drs. Bruno Stieger and Peter Meier); and $\beta$-actin (C4, Chemicon, San Francisco, CA). After incubation with HRP-conjugated secondary antibody, signals were detected by chemiluminescent substrate Supersignal West Duro (Pierce, Rockford, IL) with a Bio-Rad VersaDoc imaging system; densitrometry analysis was performed using Quantity One V4.1 software (Bio-Rad Laboratories, Hercules, CA).

**Accumulation Studies in Rat SCH.** Accumulation studies were conducted in SCH on day 4 as described previously.[31] Briefly, cells were washed twice with 0.3 mL of warm standard ($Ca^{2+}$-containing) or $Ca^{2+}/Mg^{2+}$-free HBSS with 0.38 g/L EGTA (hereafter referred to as $Ca^{2+}$-free) and incubated in the same buffer for 10 min at 37 °C to maintain or disrupt tight junctions, respectively. Subsequently, cells were incubated at 37 °C for 10 min with 0.25 mL of standard HBSS containing [$^3$H]TC, [$^3$H]RSV, or [$^3$H]$E_2$17G at 1 $\mu$M (100 nCi/mL). After 10 min, cells were washed 3× with ice-cold standard HBSS and lysed with 0.25 mL of 0.5% (v/v) Triton X-100 in phosphate-buffered saline. Samples were quantified by a Tri-Carb 3100 TR liquid scintillation analyzer (Perkin-Elmer, Waltham, MA). Transport function was normalized to the protein content of each preparation using the BCA protein assay. The biliary excretion index (BEI; %) was calculated using B-CLEAR technology (Qualyst Transporter Solutions, Research Triangle Park, NC) as follows:

$$\mathrm{BEI}\,(\%) = \frac{\mathrm{accumulation}_{\mathrm{cells+bile}} - \mathrm{accumulation}_{\mathrm{cells}}}{\mathrm{accumulation}_{\mathrm{cells+bile}}} \times 100$$

**Experimental Design.** SCH were obtained from $n = 3$ WT rats and $n = 3$ TR$^-$ rats. From each rat, sets of 3 SCH samples ("triplicates") were systematically assigned by plate location to each of the 36 combinations of virus (Ad-siBcrp, Ad-siNT, noninfected), MOI (1, 3, 5, 10), and evaluation procedure (RT-PCR, immunoblots, RSV accumulation, TC accumulation, as noted in Figures 1, 2, 3). For each level of MOI, triplicates were assigned to the three levels of virus (Ad-siBcrp, Ad-siNT, noninfected) in ratios of 1:1:1. For RT-PCR and immunoblot assays, two of the triplicate samples were measured, which provided 2 numerical assay values that were averaged together as a preliminary step for statistical analysis. For the accumulation studies of probe substrates, triplicate samples provided 3 numerical assay values that were averaged together as a preliminary step for statistical analysis. MOI of 1 was intentionally not studied by RT-PCR and immunoblots; otherwise, the experimental design produced complete data (i.e., no missing values for any assays).

**Auxiliary Experimental Designs.** Preliminary experimentation briefly explored two alternatives to the recommended knockdown system: (1) a system using Ad-siMrp2 for knockdown as an alternative to relying on TR$^-$ rat SCH, and (2) a system using Ad-siBcrp with Ad-siMrp2 for double-knockdown. SCH obtained from $n = 4$ WT rats were systematically assigned to selected combinations of three factors: virus (Ad-siMrp2, Ad-siBcrp with Ad-siMrp2, Ad-siNT, noninfected), MOI (5, 10, 15, 20), and evaluation procedure (RT-PCR, immunoblots, probe accumulation). Intentionally, the combinations studied were assigned to either triplicate or duplicate sets of SCH samples from 2, 3, or 4 rats (as noted in Supplement Figures 1, 2, and 3 in the Supporting Information); otherwise, the design produced complete data (i.e., no missing values for the assays.)

**Two-Stage Statistical Analysis Strategy.** The "dose-finding" analyses of stage 1 (Figures 1, 2, 3) explored the dose−response relationship between MOI and measures of the resulting on-target and off-target effects. For the MOI "dose" selected as optimal, stage 2 (Table 1) summarized for each of two probes, RSV and TC, the effects of suppressing Bcrp and/or Mrp2 function. Stages 1 and 2 illustrate proposed approaches for

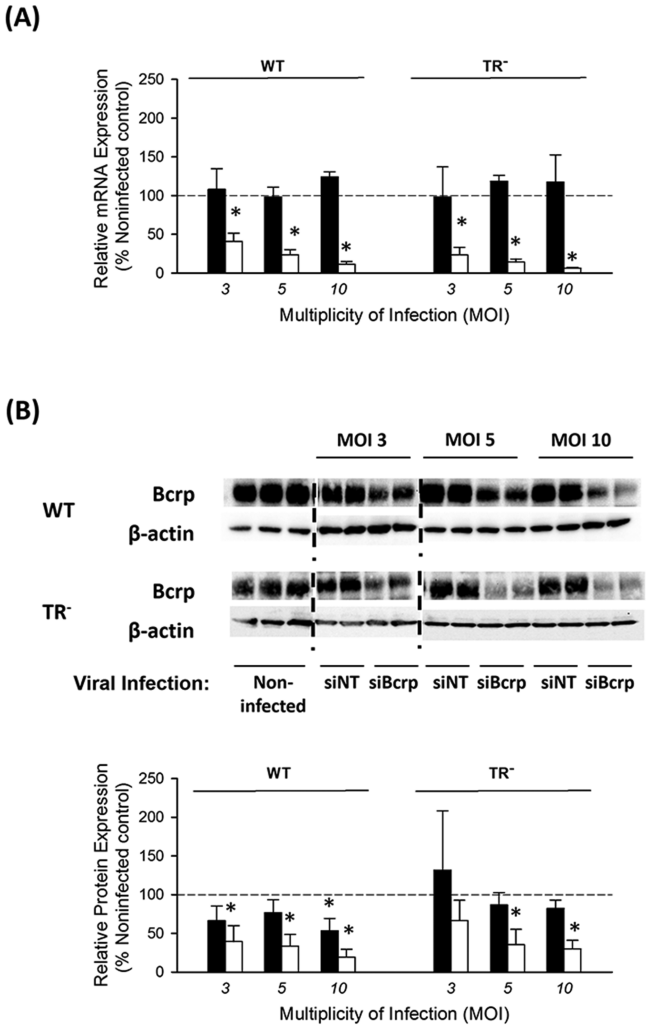


**Figure 1.** Efficient knockdown of Bcrp in WT and TR⁻ rat SCH. (A) Mean relative levels of Bcrp mRNA in SCH samples infected with Ad-siNT (solid bar) or Ad-siBcrp (open bar) at MOI of 3, 5, and 10. Each bar represents mean ± SEM of $n = 3$ rat livers. Duplicate SCH samples from each liver were analyzed; mRNA levels were expressed as a percent of the mean for noninfected hepatocytes and averaged together as a preliminary step for statistical analysis. The hypothesis testing procedure relied on a univariate repeated-measures ANOVA model for mean response as a function of MOI and virus. The model was fit separately to WT and TR⁻ rat SCH data. (B) Mean relative levels of Bcrp protein in SCH samples infected with Ad-siNT (solid bar) or Ad-siBcrp (open bar) at MOI of 3, 5, and 10. Representative blots from three independent studies are shown. $\beta$-Actin was used as the loading control for each blot. Each bar represents mean ± SEM of $n = 3$ rat livers. Duplicate SCH samples from each liver were analyzed; protein levels were expressed as a percent of the mean for noninfected hepatocytes and averaged together as a preliminary step for statistical analysis. The hypothesis testing procedure relied on a univariate repeated-measures ANOVA model for mean response as a function of MOI and virus. The model was fit separately to WT and TR⁻ rat SCH data. *, statistically significantly different from noninfected hepatocytes ($p < 0.05$).

MOI selection and characterization of the effects of impaired transporter function on the disposition of compounds of interest.

In stage 1, the extent of Bcrp knockdown (Figure 1) was evaluated in terms of Bcrp mRNA expression, and separately in terms of Bcrp protein: the primary inferential analysis of Bcrp expression relied on a univariate repeated-measures analysis of variance model (unirep-ANOVA) assuming that mean expression was a function of seven categories defined by virus [Ad-siBcrp, Ad-siNT, and noninfected (MOI = 0 only)] and MOI (3, 5, 10 for Ad-siBcrp and Ad-siNT). The model was fit to the TR⁻ rat SCH data, and separately to the WT rat SCH data yielding statistical estimates of mean expression [with 95% confidence intervals (CIs)], residual variance, and within-rat correlation. The extent of Bcrp knockdown was defined by the three mean differences between Ad-siBcrp infected (MOI = 3, 5, 10) and noninfected (MOI = 0) SCH. The null hypothesis "all three differences are exactly zero" was rejected if any of the three subhypotheses were rejected by the (modified-Bonferroni) Hochberg test procedure ($\alpha = 0.05$). The same strategy was applied to similar hypothesis tests regarding Ad-siNT. The graphical summary was computed in terms of relative expression; specifically, the values for each rat type (WT or TR⁻) were expressed as a percent of the average that was observed in that noninfected SCH samples.

Continuing stage 1, potential off-target knockdown (Figure 2) was explored for a selection of six other proteins (Oatp1a1, Ntcp, Bsep, P-gp, Mrp4, and Mrp2) using the same graphical summary method and the same inferential analysis methods that were applied to Bcrp protein expression. For each protein, the unirep-ANOVA model was used to obtain point and confidence interval estimates of mean expression as a function of five categories defined by virus [Ad-siBcrp, Ad-siNT, and noninfected (MOI = 0 only)] and MOI (5, 10 for Ad-siBcrp and Ad-siNT). The hypothesis testing strategy was as described above for Bcrp protein.

Completing stage 1, the dose–response relationship (Figure 3) between MOI and disposition of RSV and TC was explored in terms of total substrate accumulation (uptake in pmol/mg of protein) and biliary excretion index (BEI %). The analysis relied on a unirep-ANOVA model assuming that the mean was a function of nine categories defined by virus [Ad-siBcrp, Ad-siNT, and noninfected (MOI = 0 only)] and MOI (1, 3, 5, 10 for Ad-siBcrp and Ad-siNT). The model was fit to the TR⁻ rat SCH data, and separately to the WT rat SCH data, yielding statistical estimates of mean levels of response (total accumulation and BEI), residual variance, and within-rat correlation. Estimates of mean response (individual and marginal) and differences among mean marginal responses were all tabulated together with 95% CIs. The off-target effects of Ad-siNT were defined by the four mean differences between Ad-siNT infected (MOI = 1, 3, 5, 10) and noninfected (MOI = 0) SCH. The null hypothesis "all four differences are exactly zero" was rejected if any of the four subhypotheses were rejected by the Hochberg test procedure ($\alpha = 0.05$). The targeted effects of siBcrp expression were defined by the four MOI-specific mean differences between Ad-siBcrp and Ad-siNT infected SCH. The null hypothesis "all four differences are zero" was rejected if any of the four subhypotheses were rejected by the Hochberg test procedure ($\alpha = 0.05$). The results from all eight unirep-ANOVA models are summarized in a graphical illustration (Figure 3).

In stage 2, best estimates (Table 1) of probe effects attributable to impaired Bcrp function and/or absence of Mrp2 function were obtained for total substrate accumulation (uptake in pmol/mg of protein) and BEI (%) using the responses from the SCH samples that were noninfected compared to those administered virus at the MOI level selected as optimal "dose". This stage 2 analyses relied on a unirep-ANOVA model assuming mean response was a function of the six categories of virus (Ad-siBcrp, Ad-siNT, noninfected) and WT or TR⁻ status. For probes RSV and TC, the resulting estimates of category means, marginal means, and


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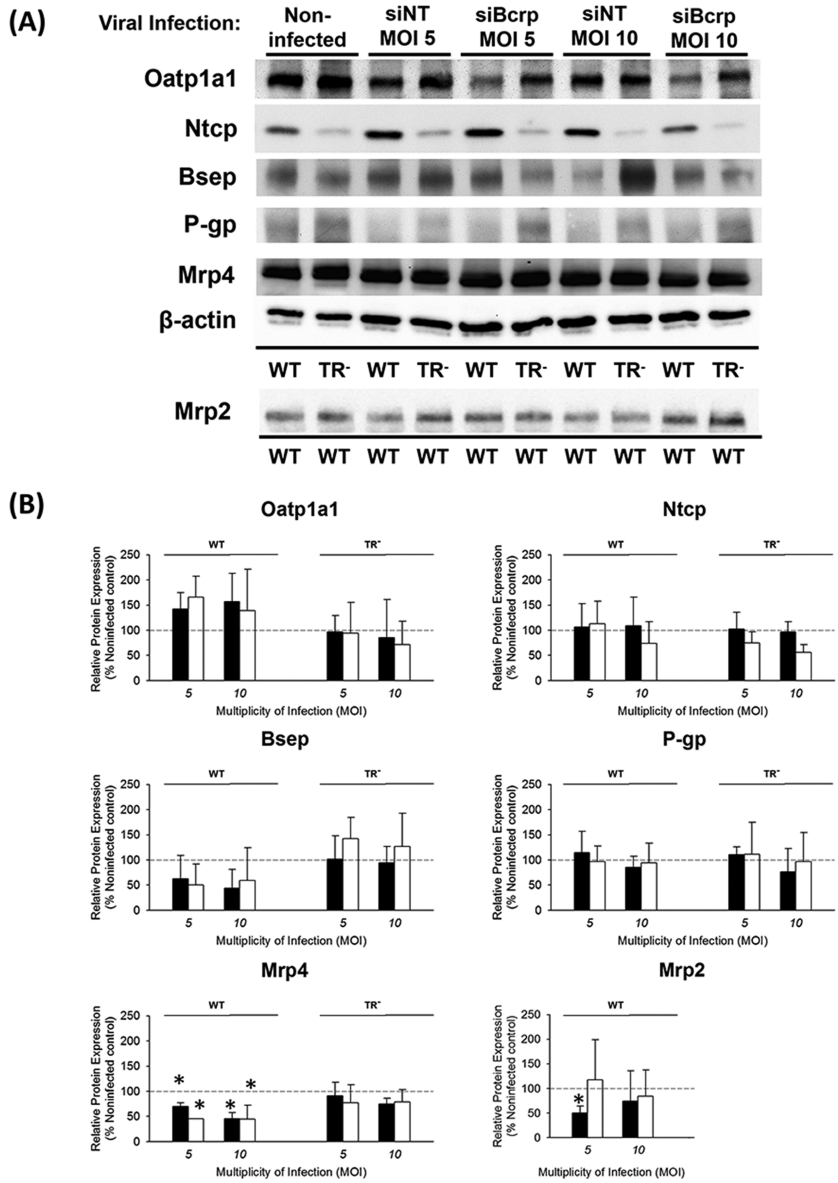


**Figure 2.** Effects of Bcrp knockdown on relative levels of Oatp1a1, Ntcp, Bsep, P-gp, Mrp4, and Mrp2 proteins in WT and TR$^-$ rat SCH infected with Ad-siNT or Ad-siBcrp. (A) Representative blots from three independent studies are shown. $\beta$-Actin was used as the loading control for each blot. Mrp2 was evaluated only in WT SCH because it is not expressed in TR$^-$ rat SCH. (B) Each bar represents mean ± SEM of $n$ = 3 rat livers. Duplicate SCH samples from each liver were analyzed; protein levels after infection with Ad-siNT (solid bar) or Ad-siBcrp (open bar) at MOI of 5 and 10 were expressed as a percent of the mean for noninfected hepatocytes and averaged together as a preliminary step for statistical analysis. The multiple hypothesis testing procedure relied on a univariate repeated-measures ANOVA model for mean response as a function of MOI and virus. The model was fit separately to WT and TR$^-$ rat SCH data. *, statistically significantly different from noninfected hepatocytes ($p < 0.05$).

contrasts thereof were tabulated with their SEs and 95% CIs. The null hypothesis tested via an $F$-test procedure ($\alpha = 0.05$) included "effects of the viruses do not depend on WT or TR$^-$ status", "no difference between the marginal means for WT and TR$^-$ rat SCH", and "no differences among three virus-specific marginal means". If and only if virus effects were detected, then an $F$-test procedure ($\alpha = 0.05$) was performed for the two subhypotheses regarding marginal means: "off-target effects are zero (siNT vs noninfected)", "targeted effects are zero (siBcrp vs siNT)". The results from all four unirep-ANOVA models are summarized in Table 1.

For stages 1 and 2, auxiliary analyses were performed to more fully explore the data and to evaluate the robustness of the main results to reasonable perturbations of the statistical modeling assumptions and methods. For example, in stage 1, unirep-ANOVA models accounting for all three factors (WT or TR$^-$ status, virus, and MOI) were evaluated and interactions were explored.

All statistical computations were performed using SAS software v9.2 (SAS Institute Inc., Cary, NC).

## RESULTS

### Targeted Knockdown of Bcrp in WT and TR$^-$ Rat SCH.

Relative to noninfected hepatocytes, mean levels of Bcrp mRNA (Figure 1A) decreased with increasing MOI of Ad-siBcrp in both WT and TR$^-$ rat SCH. For MOI 10, the mean was only 12% and 6.5% of that in noninfected WT and TR$^-$ rat SCH, respectively. In contrast, Ad-siNT infection had negligible impact on mean


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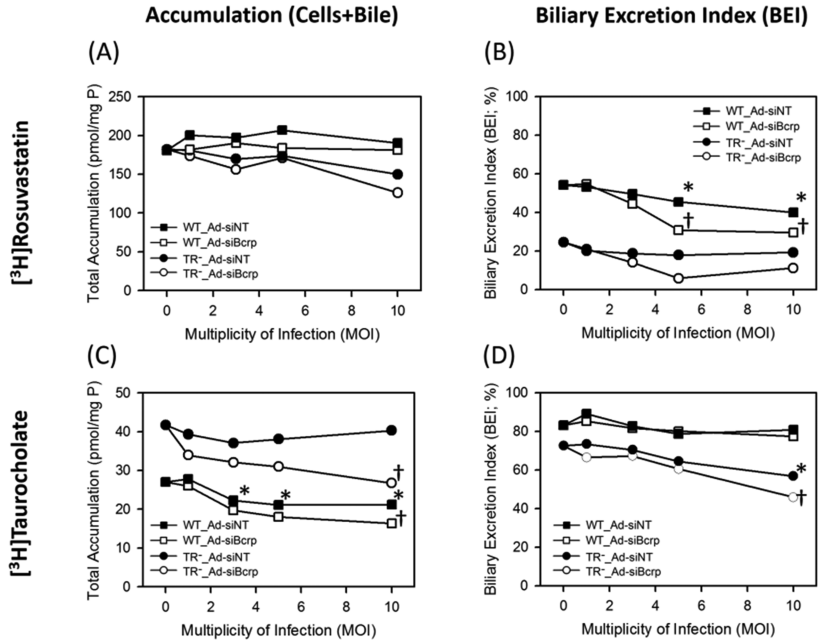


**Figure 3.** Effects of nontargeted and targeted adenoviral infection in WT and TR$^-$ SCH at increasing MOI. (A) Total accumulation and (B) biliary excretion index (BEI) of [$^3$H]rosuvastatin; (C) total accumulation and (D) BEI of [$^3$H]taurocholate. Mean values represent the responses of SCH preparations from $n$ = 3 WT (squares) and TR$^-$ (circles) rat livers, respectively, infected with nontargeted (Ad-siNT, closed symbols) or targeted (Ad-siBcrp, open symbols) adenoviral vectors. Triplicate SCH samples from each liver were measured and averaged together as a preliminary step for statistical analysis. Estimation of means and differences between means relied on a univariate repeated-measures ANOVA model for mean response as a function of MOI and virus. The model was fit separately to WT and TR$^-$ rat SCH data. *, statistically significant off-target effect [siNT vs noninfected (MOI of 0)]. †, statistically significant targeted effect (siBcrp vs siNT).

**Table 1. Total Accumulation and BEI (Mean ± SEM) of Probe Substrates at MOI = 5 vs Noninfected Control**[a]

| | total accumulation (pmol/mg of protein) | | | biliary excretion index (BEI; %) | | |
|---|---|---|---|---|---|---|
| | WT or TR$^-$ status | | | WT or TR$^-$ status | | |
| viral treatment | wild-type | TR$^-$ | LSM | wild-type | TR$^-$ | LSM |
| | | | (A) [$^3$H]Rosuvastatin | | | |
| noninfected | 181 (25) | 182 (29) | 181 (13) | 54.3 (8.2) | 24.6 (11.0) | 39.4 (4.1) |
| nontargeted (Ad-siNT) | 207 (48) | 174 (47) | 190 (14) | 45.4 (6.6) | 17.9 (12.7) | 31.7 (4.5)* |
| Ad-siBcrp | 184 (30) | 171 (45) | 177 (14) | 30.8 (7.5) | 5.9 (3.0) | 18.4 (4.5)*,† |
| LSM | 187 (18) | 180 (18) | | 44.1 (5.8) | 15.5 (5.8)# | |
| | | | (B) [$^3$H]Taurocholate | | | |
| noninfected | 27.1 (6.3) | 41.7 (11.8) | 34.4 (5) | 83.1 (5.9) | 72.6 (5.5) | 77.8 (2.7) |
| nontargeted (Ad-siNT) | 21.2 (5.7) | 38.0 (14.3) | 29.6 (5)* | 78.6 (8.3) | 64.5 (9.0) | 71.5 (3.0)* |
| Ad-siBcrp | 18.0 (5.6) | 31.0 (8.7) | 24.5 (5)*,† | 80.1 (5.5) | 60.6 (3.1) | 70.3 (3.0)* |
| LSM | 22.1 (6) | 36.9 (6) | | 79.5 (3.8) | 66.9 (3.8) | |

[a]Means were estimated via a univariate repeated-measures ANOVA model. Estimates of marginal means are least squares means (LSM) ± SEM. *$p$ < 0.05 compared to noninfected. †$p$ < 0.05 compared to nontargeted. #$p$ < 0.05 compared to wild-type.

Bcrp mRNA. Similarly, for Bcrp protein (Figure 1B) the mean level decreased with increasing MOI of Ad-siBcrp in both WT and TR$^-$ rat SCH relative to noninfected hepatocytes. In WT rat SCH, the mean decreased by 61%, 66%, and 81% with Ad-siBcrp at MOI of 3, 5, and 10, respectively. In TR$^-$ rat SCH, the mean decreased by 33%, 65%, and 70%, respectively. In contrast, Ad-siNT infection had much less impact on the mean level of Bcrp protein in TR$^-$ and WT rat SCH; however, for MOI of 10 in WT, the mean level was decreased by 47%, suggesting that off-target (nonspecific) effects of viral infection can exist at high MOI (≥10) under the conditions of the proposed SCH knockdown system.

**Off-Target Effects of Bcrp Knockdown on the Levels of Other Transport Proteins.** For TR$^-$ rat SCH, no off-target effects were detected for Oatp1a1, Ntcp, Bsep, P-gp, Mrp4, and Mrp2 proteins at MOI of 5 and 10 (Figure 2). For WT rat SCH, off-target effects (Ad-siNT vs noninfected) were detected only for Mrp4 and Mrp2 proteins. Mean levels of Mrp4 protein were decreased by 30% and 55% with Ad-siNT at MOI of 5 and 10, and by 55% and 56% with Ad-siBcrp at MOI of 5 and 10, respectively, relative to noninfected SCH samples. For Mrp2 protein, an off-target effect was statistically significant for Ad-siNT at MOI of 5, but not at MOI of 10, and not for Ad-siBcrp at either MOI level.

**Effects of Impaired Transporter Function on Probe Disposition.** Based on stage 1 analyses, MOI = 5 was selected as the optimal "dose" for stage 2 analyses of probes RSV and TC.

*RSV Total Accumulation.* Mean total accumulation of RSV was similar in WT and TR$^-$ rat SCH, and minimally altered by viral infection. Stage 1 analyses (Figure 3A) did not detect off-target effects (siNT vs noninfected), targeted effects (siBcrp vs siNT), nor any effects with increasing MOI. In stage 2 analysis (Table 1), targeted and nontargeted effects were not detected in samples administered virus at the MOI of 5.

*RSV BEI.* MOI-dependent effects of virus and WT or TR$^-$ status were observed in stage 1 analyses (Figure 3B). For WT rat SCH, a mean decrease in BEI due to off-target effects (siNT vs noninfected) was detected at MOI of 5 and 10 only, and a mean decrease in BEI due to targeted effects (siBcrp vs siNT) was detected at MOI of 5 and 10 only. For TR$^-$ rat SCH, the pattern of response was similar but no effects were statistically significant. Stage 2 analyses (Table 1) detected effects for both factors. The marginal mean for TR$^-$ was smaller than for WT rat SCH by 28.6% with 95% CI [5.8, 51.3]. The targeted and nontargeted effects were statistically significant: relative to noninfected samples, siNT reduced the marginal mean BEI by 7.8% [3.2, 12.4]; relative to siNT, siBcrp reduced the marginal mean BEI by 13.3% [7.5, 19.1]. The difference in BEI between noninfected and siBcrp was 21% [16.5, 25.7].

*TC Total Accumulation.* MOI-dependent effects were found in stage 1 analyses (Figure 3C): For WT rat SCH, the dose-dependent off-target effects were statistically significant for MOI of 3, 5, and 10. For TR$^-$ rat SCH, off-target effects were not detected. For both WT and TR$^-$ rat SCH, targeted effects were statistically significant only for MOI of 10. In stage 2 analyses (Table 1), focusing on MOI = 5, the marginal means were decreased by nontargeted and targeted effects: relative to noninfected samples, siNT reduced the marginal mean by 4.8 pmol/mg of protein [1.9, 7.7]; relative to siNT, siBcrp reduced the marginal mean by 5.1 pmol/mg of protein [1.4, 8.8]. The difference between noninfected and siBcrp was 9.9 pmol/mg of protein [7.0, 12.8].

*TC BEI.* In stage 1 analyses (Figure 3D) for TR$^-$ rat SCH, off-target effects and targeted effects were statistically significant at an MOI of 10. In stage 2 analyses (Table 1), an off-target effect was evident: relative to noninfected samples, siNT reduced the marginal mean BEI by 6.3% [2.5, 10.1]. The targeted effect was not detected, as in comparison to siNT, siBcrp reduced the marginal mean BEI by only 1.2% [−3.7, 6.1]; however, the difference in BEI between noninfected and siBcrp, 7.5% [3.7, 11.3], was statistically significant. The difference in BEI between TR$^-$ and WT, 12.6% [−2.1, 27.3], was not statistically significant.

*Sensitivity Analyses and Exploratory Analyses.* The main results of stage 1 and stage 2 analyses were robust to perturbations of the modeling assumptions (e.g., variance homogeneity across WT and TR$^-$ rat SCH). In stage 1, a MOI-by-WT or TR$^-$ interaction was observed in 3-factor models for the analyses of TC BEI ($p$ = 0.0038) and RSV total accumulation ($p$ = 0.0460); however, inclusion or exclusion of interactions and commonality assumptions yielded negligible changes in the main results of interest (data not presented).

**Ancillary Study of Knockdown of Mrp2 in WT Rat SCH.** Infection with Ad-siMrp2 decreased mean relative levels of Mrp2 protein by 45%, 79%, and 78% at MOI of 5, 10 and 15, respectively, compared to noninfected SCH (Supplement Figure 1 in the Supporting Information). Infection with Ad-siNT decreased the mean by 39% at MOI of 10, suggesting that some off-target effects may exist for large MOI. In contrast, Ad-siNT or Ad-siMrp2 at MOI of 5, 10, and 15 had little influence on the mean relative level of Bcrp protein.

Mean total accumulation of $E_2$17G (Supplement Figure 2A in the Supporting Information) showed little evidence of targeted or off-target effects. Mean BEI values for $E_2$17G (Supplement Figure 2B in the Supporting Information) appeared to decrease slightly with increasing MOI. Mean total accumulation of TC (Supplement Figure 2C in the Supporting Information) was decreased by Ad-siNT and Ad-Mrp2 at MOI of 10, suggesting again that off-target effects become more prevalent at MOI $\geq$ 10. Mean BEI values for TC (Supplement Figure 2D in the Supporting Information) were influenced least by viral infection. Due to the off-target effects on TC total accumulation at an MOI of 10, an MOI of 5 was selected for the double knockdown study.

**Ancillary Study of Double Knockdown of Mrp2 and Bcrp in WT Rat SCH.** To suppress both Mrp2 and Bcrp (Supplement Figure 3 in the Supporting Information), SCH were infected with Ad-siNT at MOI of 10, Ad-siMrp2 and Ad-siBcrp (MOI of 5 each; total MOI = 10), or not infected. The targeted effects (double knockdown vs Ad-siNT) decreased the mean relative levels of Mrp2 protein and Bcrp protein by 67% and 64%, respectively, relative to noninfected control. However, off-target effects also were observed: Ad-siNT infection at MOI of 10 decreased the mean levels of Mrp2 protein and Bcrp protein by 47% and 30%, respectively, relative to noninfected SCH samples. MOI of 10 also induced off-target effects in Bcrp knockdown SCH described elsewhere in this report; at MOI = 10, Ad-siNT decreased mean relative levels of Bcrp (Figure 1B) and Mrp4 (Figure 2) in WT rat SCH, and altered hepatobiliary disposition of probe substrates [i.e., TC total accumulation in WT SCH (Figure 3C), RSV BEI in WT SCH (Figure 3B), and TC BEI in TR$^-$ SCH (Figure 3D)].

## ■ DISCUSSION

Protein knockdown in cultured primary hepatocytes is challenging because it is difficult to reach high transfection efficiency using conventional transfection reagents. Our laboratory previously had established an efficient and specific Bcrp knockdown system in WT rat SCH in 6-well plates using adenoviral vectors to deliver shRNA into hepatocytes.[28] In the present study, that work was extended to establish an *in vitro* system exhibiting impaired function of multiple specific transport proteins in the 24-well plate format. Scaling from 6-well to 24-well plates enabled more efficient use of hepatocytes and other resources required for the study. Initially, double knockdown of both Mrp2 and Bcrp was attempted. However, off-target effects in 24-well rat SCH were noted at the higher MOI required to knock down multiple transport proteins. Because an MOI of 5 was required for efficient knockdown of each transport protein (Mrp2 and Bcrp), the combination of two different shRNA targeting different transport proteins required an MOI of 10, at which off-target effects were prevalent. Use of a tandem plasmid vector that expresses two different shRNA to facilitate the simultaneous double knockdown of genes has been applied in stable cell lines.[32] However, plasmid DNA has low transfection efficiency into primary hepatocytes,[27] and adenoviral vectors that similarly express tandem expression shRNA are not commercially available. Therefore, efficient double knockdown of transport proteins using the adenoviral vector approach necessitated a higher viral load in hepatocytes. In order to circumvent this problem, Mrp2-deficient TR$^-$ rat SCH were employed in combination with Bcrp knockdown using adenoviral infection of shRNA targeting Bcrp.

To validate this *in vitro* system, the impact of Bcrp knockdown on the hepatobiliary disposition of RSV and TC was examined in

the absence and presence of functional Mrp2. RSV and TC were selected as probe substrates because different mechanisms dominate their hepatocellular uptake and biliary excretion. The results, for a range of MOI "doses" with multiple viral vectors (siNT vs siBcrp or siMrp2), demonstrated the importance of optimizing the system when an RNAi approach is employed to knock down transport proteins. This approach allows greater confidence in identification of off-target effects and interactions among factors that might otherwise have been dismissed as spurious if evaluated at only one level of MOI. This report demonstrates the use of a two-stage statistical analysis: stage 1 evaluated targeted and off-target effects for a range of MOI in order to identify dose−response relationships and select an optimal MOI "dose" for the system. Stage 2 summarized for each of two probes, RSV and TC, the effects of impaired Bcrp and/or Mrp2 function. Results of stages 1 and 2 analyses demonstrated MOI selection and characterization of the effects of impaired transporter function on the disposition of compounds of interest. Since changes in protein levels may not always translate to changes in protein activity, use of relevant probe substrates (positive and negative controls; TC and RSV in this study) to assess changes in protein activity is strongly recommended.

The MOI of 5 was chosen as the "dose" for use in stage 2 analyses of the relative contributions of Mrp2 and Bcrp to the hepatobiliary disposition of the probe substrates. The main considerations in choosing the optimal MOI "dose" were the MOI-dependent patterns of targeted and off-target effects observed in stage 1. For example, targeted effects on RSV BEI were observed at MOI of 5 and 10. In analysis of total accumulation of TC, off-target effects were evident at MOI of 3, 5, and 10. At MOI of 10, off-target effects were maintained or increased based on immunoblots and TC BEI. These data suggest that an MOI of 5 is a better choice than an MOI of 3 or 10.

In stage 1 analyses, some evidence of interactions between MOI and WT or TR$^-$ status were observed in auxiliary unirep-ANOVA models for TC BEI and RSV total accumulation. Such interactions are biologically plausible because loss of Mrp2 function (as in TR$^-$ rats) may alter the regulatory machinery of the cell due to accumulation of endogenous substances such as bilirubin and bile acid conjugates. This study is consistent with the premise that altered function of individual transport proteins does not occur in isolation. Rather, as a result of overlapping substrate specificity and reliance on multiple mechanisms for vectorial transport from blood to bile, a complex network of cellular regulation is perturbed along with transport function, leading to compensatory changes. If so, it is advantageous to use an organ-specific *in vitro* model system, such as SCH, which recapitulates the relevant disposition, regulatory mechanisms, and interplay expected *in vivo*.

At MOI of 5, viral infection had minimal effects on RSV total accumulation. The nontargeted effect (siNT vs noninfected) statistically significantly decreased the mean BEI of RSV, and the targeted effect (siBcrp vs siNT) further reduced the mean BEI of RSV, consistent with the statistically significant mean decrease in Bcrp protein attributed to siBcrp. Mean BEI of RSV was decreased further by TR$^-$ status, suggesting an additive effect of targeted Bcrp knockdown and loss of Mrp2 function. Since Bcrp expression and function are decreased significantly in TR$^-$ rat SCH,[33] reduced RSV BEI in Bcrp knockdown TR$^-$ rat SCH resulted from the combined effects of lack of Mrp2, an inherent decrease in Bcrp expression, and targeted knockdown of Bcrp. This is consistent with the known role of Bcrp and Mrp2 in the biliary excretion of RSV *in vivo* in rats.[17]

For TC, mean total accumulation and mean BEI values were decreased after viral infection relative to noninfected hepatocytes. TC is a bile acid that is not transported by Bcrp and Mrp2. This finding may be attributed to off-target effects of viral infection on uptake and efflux pathways involved in the hepatobiliary disposition of bile acids. If so, Ntcp function or Bsep function might also be altered by viral infection. Although the immunoblot analysis failed to detect off-target effects for Ntcp and Bsep proteins, this system should be used with caution when testing the hepatobiliary disposition of Ntcp and/or Bsep substrates. Since RSV is transported by human NTCP, but not by rat Ntcp,[34] RSV uptake was not influenced by potential off-target effects on Ntcp function in this system.

Viral infection appeared to exert differential off-target effects on individual transport proteins. Mean levels of Mrp4 protein were decreased in siNT- and siBcrp-infected hepatocytes at MOI of 5 and 10. Viral infection also decreased mean levels of Mrp2 protein in siNT-infected rat SCH, whereas off-target effects of viral infection were not detected on other transport proteins. Notably, off-target effects on Mrp4 were observed in WT, but not in TR$^-$ SCH. Mrp4 is known to be induced by constitutive androstane receptor (CAR),[35] which is activated by bilirubin and bile acids.[36,37] Thus, it is plausible that accumulation of organic anions such as bilirubin and bile acids in TR$^-$ rat hepatocytes due to lack of Mrp2 prevents viral infection-mediated down-regulation of Mrp4 through nuclear receptor regulation. However, further studies are needed to investigate the mechanism(s) of differential downregulation of Mrp4 in WT and TR$^-$ rats. RSV undergoes biliary and basolateral efflux to a quantitatively similar extent; MRP4 contributes to basolateral efflux of RSV.[38] However, RSV total accumulation was not altered by decreased Mrp4 protein expression in siNT- and siBcrp-infected WT rat SCH, suggesting minimal changes in Mrp4 function or the presence of other basolateral efflux transport proteins that can compensate for impaired Mrp4 function.

Recombinant adenovirus has been used widely as a gene delivery vector because of its high infection efficiency and high transgene capacity compared to other viral vector systems (i.e., lentivirus, retrovirus).[27] Thus, it provides a useful tool to deliver siRNA to primary cells, for which gene delivery is challenging. Recently, Hollingshead et al. reported a high-throughput gene silencing method in mouse SCH using transfection reagents.[39] To increase transfection efficiency, a "reverse" transfection method was employed that initiated the transfection of suspended hepatocytes prior to plating. This approach resulted in a significant decrease in mRNA levels of Cyp3a11/13. Although this approach provides a high-throughput method for functional studies, the expression and function of proteins-of-interest, as well as potential off-target effects, need to be investigated further.

There is increasing evidence that membrane transport proteins play an important role in the pharmacokinetics of many drugs. Effects of altered function of uptake transporters often are reflected in systemic drug exposure. However, it is more challenging to assess the consequences of altered function of efflux transporters because changes in cellular (e.g., hepatocyte) exposure, which may be important in predicting efficacy and toxicity, may not lead to changes in systemic exposure.[40−42] The SCH model is an experimental tool that retains hepatic transport and metabolic capabilities, and provides information about

hepatic exposure (intracellular concentration), systemic exposure (medium concentration), and biliary excretion (BEI, biliary clearance) in isolation from other organs. The current study assessed the utility of RNAi in SCH from hepatocytes lacking specific transport proteins (e.g., Mrp2-deficient $TR^-$ rat hepatocytes), as an *in vitro* tool to predict altered accumulation or disposition of drugs when multiple efflux transporters are impaired. This approach requires initial efforts for optimization, but once optimized, it has potential utility for rapid screening of a number of compounds. Concerns regarding possible off-target effects should be addressed further with validation of the system using additional compounds. Recently, Mrp2- and Bcrp-knockout rats have been shown to possess modest compensatory changes in expression of ADME-related genes, providing a useful *in vivo* system to explore the contribution of these transporters to drug disposition.[43] However, results obtained from preclinical species may not necessarily translate to humans because of species differences in transport protein expression, regulation, and function. To address this question, RNAi also can be applied to human SCH and future technologies in development (e.g., induced pluripotent stem cells, bioengineered culture systems such as HepatoPac and the Liver Chip) to assess species differences in transporter function and altered drug disposition. However, for knockdown of multiple transport proteins in human SCH, alternative approaches (e.g., tandem plasmid vector that can express two different siRNA in one vector) should be developed to minimize potential off-target effects.

## ■ ASSOCIATED CONTENT

### Supporting Information

Supporting figures including (1) Mrp2 and Bcrp protein levels, (2) hepatobiliary disposition of [$^3$H]estradiol-17$\beta$-D-glucuronide ($E_2$17G) and [$^3$H]taurocholate (TC) in Mrp2 knockdown WT rat SCH, and (3) Mrp2 and Bcrp protein levels in Mrp2/Bcrp double knockdown WT rat SCH. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION


### Corresponding Author

*E-mail: kbrouwer@unc.edu. Tel: 919-962-7030. Fax: 919-962-0644.

### Present Addresses

[‖]W.Y.: Department of Pharmaceutical Sciences, College of Pharmacy, The University of Oklahoma Health Sciences Center, Oklahoma City, OK.

[#]UNC Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, 311 Pharmacy Lane, CB #7569, Kerr Hall, Chapel Hill, NC 27599-7569.

### Author Contributions

[‡]Authors contributed equally to this work.


### Notes

The authors declare the following competing financial interest(s): Dr. Kim Brouwer is co-inventor of the sandwich-cultured hepatocyte technology for quantification of biliary excretion (B-CLEAR) and related technologies, which have been licensed exclusively to Qualyst Transporter Solutions. Dr. Brouwer chairs the Scientific Advisory Board for Qualyst Transporter Solutions.

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## ■ ABBREVIATIONS USED

ABC, ATP-binding cassette; BCRP/Bcrp, breast cancer resistance protein; BEI, biliary excretion index; BSEP/Bsep, bile salt export pump; CI, confidence interval; HBSS, Hanks' balanced salt solution; MRP2/Mrp2, multidrug resistance-associated protein 2; P-gp, P-glycoprotein; RNAi, RNA interference; SCH, sandwich-cultured hepatocytes; shRNA, short hairpin RNA; $TR^-$, Mrp2-deficient Wistar rats; WT, wild-type rats; Ad-siBcrp, adenoviral vectors expressing shRNA targeting rat Bcrp; Ad-siMrp2, adenoviral vectors expressing shRNA targeting rat Mrp2; Ad-siNT, adenoviral vectors expressing a nontargeting shRNA; RSV, rosuvastatin; TC, taurocholate; unirep-ANOVA, univariate repeated-measures analysis of variance (a linear statistical model)

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