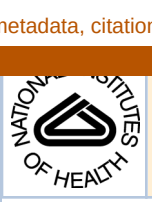

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Knocking Down Breast Cancer Resistance Protein (Bcrp) by Adenoviral Vector-Mediated RNA Interference (RNAi) in Sandwich-Cultured Rat Hepatocytes: A Novel Tool to Assess the Contribution of Bcrp to Drug Biliary Excretion

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Abstract

BCRP transports numerous drugs/derived metabolites and toxins, and exhibits overlapping substrate specificity with P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 (MRP2). Assessing the contribution of BCRP to drug/metabolite biliary excretion in the intact hepatocytes remains a challenge. Current studies were designed to develop a novel *in vitro* tool to specifically assess the contribution of Bcrp to drug biliary excretion. Adenoviral vectors expressing short hairpin (sh) RNA targeting Bcrp (Ad-si01Bcrp) or a non-target control (Ad-siNT) were packaged and infected into sandwich-cultured rat hepatocytes (SCRH). Protein levels were detected by immunoblot. Biliary excretion index (BEI) and *in vitro* biliary clearance ($Cl_{biliary}$) of nitrofurantoin (BCRP substrate) and digoxin (P-gp substrate) were compared among non-infected, Ad-siNT- and Ad-si01Bcrp-infected cells in SCRH. shRNA targeting Bcrp efficiently knocked down Bcrp in SCRH, while levels of other transport proteins (P-gp, Mrp2, Bsep, Mrp4 and Oatp1a1) were unaffected. In SCRH exhibiting Bcrp knockdown, cellular accumulation of nitrofurantoin was increased markedly and nitrofurantoin BEI and *in vitro* $Cl_{biliary}$ were decreased to 11% and 14% of control, respectively. Digoxin values were unaffected by knockdown of Bcrp. Results indicated that Bcrp contributed predominantly to nitrofurantoin biliary excretion, but played a negligible role in digoxin biliary excretion. In summary, Bcrp knockdown in SCRH is the first *in vitro* model utilizing intact hepatocytes to assess the contribution of Bcrp to the biliary excretion of drugs. This approach may be useful in predicting drug-drug interactions in biliary excretion and the consequence of impaired BCRP function on the hepatic exposure of drugs/derived metabolites.

Keywords

Breast Cancer Resistance Protein (BCRP); RNA interference (RNAi); biliary excretion; sandwich-cultured hepatocytes; nitrofurantoin

INTRODUCTION

Breast cancer resistance protein (BCRP; ABCG2), a member of the ATP-binding cassette (ABC) transporter family, is a membrane glycoprotein localized to the apical region in

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polarized cell types[1]. BCRP is abundantly expressed in a variety of healthy tissues including the liver, intestinal epithelia, placenta and blood-brain barrier. Many anticancer drugs, cytotoxic drugs and partially detoxified metabolites, toxins, carcinogens in food products, and endogenous compounds are BCRP substrates[2, 3]. Modulation of BCRP expression and function may affect the pharmacokinetics of some drugs. For example, the single-nucleotide polymorphism in *ABCG*2 C421A (Q141K) is associated with decreased BCRP protein expression and function *in vitro*[4, 5], and altered drug pharmacokinetics in clinical studies (elevated plasma concentrations of diflomotecan after intravenous administration, and of topotecan and rosuvastatin after oral administration)[6–8]. Gender differences in expression of BCRP/Bcrp in humans and mice have been reported, and lower Bcrp expression in female mice and humans was associated with changes in drug pharmacokinetics[9, 10]. These data suggest that BCRP may play a clinically important role in the disposition of some drugs.

The ABC transport protein family members BCRP, multidrug resistance protein (MDR) 1 (P-gp), and multidrug resistance-associated protein 2 (MRP2) frequently have overlapping substrate profiles[2, 3]. Data obtained from membrane vesicular transport studies and vectorial transport assays in MDCK II cells often indicate that one drug can be transported by multiple transport proteins[11, 12]. Assessing the contribution of individual transport proteins to drug excretion is of great importance in identifying the major transport protein for a particular drug when multiple transport proteins are involved. Such information is required to accurately predict the clinical significance of altered hepatic transport function caused by disease states, drug interactions or polymorphisms[2, 13].

Several loss-of-function strategies have been applied to assess the contribution of BCRP to drug elimination. One strategy is to use “specific” inhibitors of BCRP; however, BCRP inhibitors identified to date[14–16] frequently interact with other transport proteins depending on the concentration. For example, GF120918 inhibits both BCRP and P-gp[15, 17]; fumitremorgin C (FTC) and its analogue Ko134 inhibit BCRP/Bcrp1, but also exhibit low inhibitory activity toward P-gp and MRP1[14]. *Abcg2*-knockout mice are a valuable *in vivo* tool to elucidate the contribution of Bcrp to drug pharmacokinetics, and the importance of Bcrp in the biliary excretion of many compounds in mice[11, 18, 19]. However, such *in vivo* studies are not high-throughput, and assessment of the contribution of Bcrp to the biliary excretion of drugs in other preclinical species and humans has been hampered by the lack of a Bcrp-deficient *in vivo* model in species other than mice. To date, an *in vitro* model using intact hepatocytes to specifically assess the contribution of BCRP to the biliary excretion of drugs has not been developed.

Sandwich-cultured rat hepatocytes (SCRH) exhibit functional metabolic activity and proper localization of basolateral and canalicular transport proteins, thus serving as a useful *in vitro* model to evaluate the biliary excretion of drugs and derived metabolites[20–22]. RNA interference (RNAi) leads to post-transcriptional, sequence-specific gene silencing and is a powerful tool for studying the loss-of-function effect of genes. Previously, our lab used synthetic small interfering RNA (siRNA) targeting Mrp2 and Mrp3 to study the contribution of these transport proteins to fluorescent 5 (and 6)-carboxy-2’,7’-dichlorofluorescein (CDF) hepatobiliary disposition[23]. Adenoviral vector delivery of siRNA typically results in high infection efficiency, and has been used widely to mediate RNAi in primary cells[24, 25].

The present study was designed to establish a robust *in vitro* method to specifically assess the contribution of Bcrp to the biliary excretion of drugs. Adenoviral vector-mediated RNAi was applied to SCRH in order to knock down Bcrp expression and assess the contribution of Bcrp to the biliary excretion of nitrofurantoin (BCRP/Bcrp substrate[26]) and digoxin (P-gp substrate[27, 28]).

MATERIAL AND METHODS

Chemicals

Insulin/transferrin/selenium (ITS+) and Matrigel™ were purchased from BD Biosciences (Bedford, MA). Dexamethasone, digoxin and nitrofurantoin were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). [^{3}H] Digoxin (40 Ci/mmol; >97% pure) was obtained from Perkin Elmer Life Sciences (Boston, MA) and CDF diacetate was purchased from Molecular Probes (Eugene, OR). N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918) was a gift from GlaxoSmithKline (Research Triangle Park, NC).

Packaging of Recombinant siRNA-Expressing Adenoviral Vectors

siRNA sequences targeted the rat Bcrp gene at positions 288–306 (si01Bcrp) and 1052–1070 (si02Bcrp) relative to the start codon; a non-target siRNA (siNT) control sequence (TAAGGCTATGAAGAGATAC) was designed to have no gene targets in human, mouse or rat cells. All siRNA sequences were purchased from Darmacon (Chicago, IL). The Clontech Adeno-X™ ViralTrak DsRed-Express Promoterless Expression System 2 (Clontech Laboratories, Mountrain View, CA) was used for packaging the recombinant adenoviral vectors that expressed short hairpin (sh) RNAs. The titer of adenoviral vectors was determined by Adeno-X Rapid Titer Kit (Clontech Laboratories, Mountrain View, CA). The adenoviral vectors that expressed siNT, si01Bcrp or si02Bcrp were designated as Ad-siNT, Ad-si01Bcrp and Ad-si02Bcrp, respectively. After viral infection, DsRed-fluorescent proteins were constitutively expressed from these adenoviral vectors, infection efficiency of adenoviral vectors was examined, and digital images were captured on a Zeiss Axiovert 200TV inverted phase contrast microscope (Carl Zeiss Inc., Thornwood, NY).

Isolation and preparation of SCRH

Hepatocytes were isolated from male Wistar rats (200–325g; Charles River Laboratory, Raleigh, NC) using a two-step collagenase perfusion method as described previously[20, 21]. Briefly, 1.75×10^6 cells were seeded onto six-well Biocoat™ pre-coated culture plates (BD Bioscience) in seeding medium [phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, nonessential amino-acids, L-glutamine, penicillin/streptomycin, 4 μg/mL insulin and 1 μM dexamethasone] and incubated at 37°C in a humidified incubator with 95% air/5% CO_2. Fresh seeding medium was replaced 2–6 h post-seeding. In some experiments, after changing seeding medium, cells were infected overnight with Ad-siNT, Ad-si01Bcrp or Ad-si02Bcrp at multiplicity of infection (MOI) of 20. Approximately 24 h post-seeding, cells were overlaid with Matrigel™ (BD Bioscience) at a final concentration of 0.25 mg/mL in DMEM containing 0.1 μM dexamethasone and ITS$^+$™ premix (feeding medium); culture medium was replaced every 24 h for the experiments.

Immunoblots

Cells were washed once with Hanks' Balanced Salt Solution (HBSS), and then resuspended in lysis buffer containing 1% SDS, 1 mM EDTA and Complete cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL). Whole-cell lysates (30 μg) were resolved on NuPAGE 4 to 20% Bis-Tris gel (Invitrogen, Carlsbad, CA) and the proteins were transferred to Polyvinylidene difluoride (PVDF) membranes. After blocking in 5% milk-Tris-buffered saline with Tween 20 (TBST) for 30 min, blots were incubated overnight at 4°C with the following antibodies: Mrp2 (M2III-6), Mrp4 (M4I-10), P-gp (C-219) and Bcrp (BXP-53) (Alexis Biochemicals, San Diego, CA); Oatp1a1 (K10, kind gift from Dr. Peter Meier); Bsep (K44, kind gift from Drs.

Bruno Stieger and Peter Meier) and β-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, densitometry analysis was performed using Quantity One v4.1 software (Bio-Rad Laboratories, Hercules, CA).

Accumulation Studies in SCRH

Experiments were performed as described previously[21, 29]. Briefly, SCRH were rinsed twice with 2 mL of standard HBSS and pre-incubated in 2 mL of either Ca^{2+}-free HBSS (in order to open the tight junctions and disrupt the canalicular networks) or standard HBSS for 10 min at 37°C. Subsequently, cells were incubated for 10 min at 37°C in 1.5 mL standard HBSS containing [^{3}H] digoxin (1μM, 100 nCi) or nitrofurantoin (5 μM) in the presence or absence of GF120918 (2 μM), an inhibitor of both P-gp and Bcrp[15, 17, 30]. After washing 3 times with ice-cold standard HBSS, cells were lysed either with 1 mL of ice-cold 0.5% Triton-X 100 in phosphate buffered saline (PBS) (digoxin studies) or methanol/water (70/30, v/v) (nitrofurantoin studies). Drug accumulation in cells + bile canaliculi (BC) (SCRH pre-incubated in standard HBSS) and cells (SCRH pre-incubated in Ca^{2+}-free HBSS) was determined by liquid scintillation spectrometry for digoxin (Packard Tricarb, Packard Corp., Meriden, CT), or by LC/MS/MS for nitrofurantoin. Accumulation, normalized to protein concentration, was corrected for nonspecific binding by including a blank plate (Biocoat™ plus Matrigel™ overlay). Due to incompatibility of the protein assay with methanol, the average protein concentration for standard HBSS or Ca^{2+}-free HBSS incubations in the same liver preparation was used to normalize the protein content for nitrofurantoin studies.

CDF excretion in SCRH

Experiments were performed as described previously[23]. Briefly, cells were rinsed twice with standard HBSS and incubated with 2 μM CDF diacetate for 10 min at 37°C. After rinsing three times with cold HBSS, CDF fluorescence images were examined, and digital images were captured on a Zeiss Axiovert 200TV inverted phase contrast microscope.

LC/MS/MS Analysis of Nitrofurantoin

SCRH were harvested and deproteinized with 1ml of methanol/water (70/30, v/v) followed by sonication and centrifugation at 4°C (12,000 ×g) for 10 min. The supernatant (20 μL) was mixed with 100 μL methanol and water (3.8:1) containing internal standard (ethyl warfarin, 10mM). A Shimadzu solvent delivery system (Columbia, MD) and a Leap HTC Pal thermostated autosampler (Carrboro, NC) connected to an Applied Biosystems API 4000 triple quadruple mass spectrometer with a Turbo Spray ion source (Applied Biosystems, Foster City, CA) were used for analysis. Tuning, operation, integration and data analysis were performed in negative mode using multiple reactions monitoring (Analyst software v.1.4.1, Applied Biosystems). Separation was accomplished using an Aquasil C18, 50×2.1 mm column, with a 5 μm particle size (ThermoElectron, San Jose, CA). The injection volume was 20 μL at a flow rate of 0.75 ml/min Initial gradient conditions (100% 10 mM ammonium acetate aqueous solution) were held for 0.75 min. From 0.75 min to 1.39 min, the mobile phase composition increased linearly to 40% methanol and the eluent was directed to the mass spectrometer. At 3.3 min, the methanol composition was increased to 90%. The flow was held at 90% methanol until 4 min. At 4 min, the column was equilibrated with 100% 10 mM ammonium acetate aqueous solution. The total run time, including equilibration, was 5 min per injection. Eight point calibration curves (2–1000 nM) were constructed based on peak area ratios of analyte and internal standard using the following transitions: nitrofurantoin (236.8→151.8) and ethyl warfarin (320.8→160.9). All points on the curves back-calculated to within ±15% of the nominal value.

Data Analysis

The accumulation (pmol/mg protein), biliary excretion index (BEI; %) and *in vitro* biliary clearance ($Cl_{biliary}$) (ml/min/kg) were calculated in hepatocytes using B-CLEAR® technology (Qualyst, Inc., Raleigh, NC) based on the following equations[29]:

$$BEI = \frac{Accumulation_{cells+BC} - Accumulation_{cells}}{Accumulation_{cells+BC}} \times 100$$

$$In\ vitro\ Cl_{biliary} = \frac{Accumulation_{cells+BC} - Accumulation_{cells}}{AUC_{medium}}$$

AUC_{medium} was determined as the product of the incubation time and the medium concentration. The concentration of drug in the medium was defined as the initial substrate concentration in the incubation medium, since the medium concentrations at the beginning and end of the incubation did not differ by more than 10%. The *in vitro* $Cl_{biliary}$ (ml/min/mg protein) was scaled to kilogram of body weight assuming the following: 200 mg protein/g of rat liver tissue and 40 g of rat liver tissue/kg of body weight[31].

Statistical Analysis

Data are expressed as mean and S.E.M. or S.D., as appropriate. Statistical comparisons were performed with SigmaStat (SPSS Inc., Chicago, IL). Statistical significance was evaluated with one-way analysis of variance (ANOVA); data shown in Figures 3 and 4 were further analyzed with Dunnett's test. In all cases, $p<0.05$ was considered statistically significant.

RESULTS

High Infection Efficiency of Adenoviral Vectors in SCRH

The infection efficiency of adenoviral vectors that expressed siRNA targeting Bcrp (Ad-si01Bcrp and Ad-si02Bcrp) or non-target control (Ad-siNT) in SCRH was examined in initial studies. DsRed-fluorescent proteins were readily detected 48 hours post infection, and could be detected throughout the entire culture time. As shown in Fig. 1A, in day 4 SCRH, more than 90% of the cells were infected by Ad-siNT and Ad-si01Bcrp at multiplicity of infection (MOI) of 20. Similar infection efficiency was achieved with Ad-si02Bcrp at MOI of 20 (data not shown).

Subsequent studies were carried out to determine whether adenoviral infection affected bile canalicular network formation and integrity. CDF diacetate passively diffuses into cells, where it is hydrolyzed to CDF and excreted into bile canalicular networks[21]. As shown in Fig. 1B, after a 10-min incubation with CDF diacetate followed by three rinses, CDF was detected in bile canalicular networks in non-infected (None) as well as adenoviral vector-infected cells (Ad-siNT and Ad-si01Bcrp). Hepatocytes infected with Ad-si02Bcrp exhibited a similar pattern of CDF excretion and accumulation in canalicular networks (data not shown). These results indicated that adenoviral vectors at MOI of 20 efficiently infected SCRH and did not appear to alter canalicular network formation or functional integrity of SCRH.

Efficient and Specific Knockdown of Bcrp in SCRH

As shown in Fig. 2A, in day 4 SCRH, protein levels of Bcrp in Ad-si01Bcrp-infected cells were markedly reduced; the relative Bcrp protein levels (Bcrp levels normalized by actin) in Ad-si01Bcrp-cells were ~30% of those in control (Ad-siNT-infected cells). In day 6 SCRH, Bcrp protein levels in Ad-si01Bcrp-infected cells were further decreased with only a trace amount of protein detected (~5% of control). Such sustained knockdown of Bcrp lasted at least until day 10. Ad-si02Bcrp resulted in knockdown of Bcrp similar to that of Ad-

si01Bcrp (data not shown). These results indicated that both siRNA sequences targeting Bcrp (si01Bcrp and si02Bcrp) resulted in potent and progressive knockdown of Bcrp expression. There was no obvious difference in Bcrp expression levels between non-infected and Ad-siNT-infected cells in day 4, day 6 or day 10 SCRH. There was no apparent difference in the expression levels of other canalicular transport proteins (P-gp, Mrp2 and Bsep), or basolateral transport proteins (Mrp4 and Oatp1a1) among non-infected (None), Ad-siNT-, or Ad-si01Bcrp-infected day 6 (Fig. 2B) or day 4 (data not shown) SCRH. Thus, adenoviral vector-mediated RNAi efficiently and specifically knocked down Bcrp expression in SCRH. Because Ad-si02Bcrp exhibited similar Bcrp knockdown efficiency and specificity as Ad-si01Bcrp (data not shown), Ad-si01Bcrp was used for subsequent functional studies in SCRH.

Knockdown of Bcrp Decreased Nitrofurantoin Biliary Excretion in SCRH

Accumulation of nitrofurantoin (10 min incubation in standard HBSS) in SCRH was concentration-dependent and appeared to be linear over the concentration range of 2 to 50 μM (Fig. 3A); 5 μM was used in subsequent uptake studies in SCRH. In order to address the contribution of Bcrp to the overall biliary excretion of nitrofurantoin, accumulation of nitrofurantoin in cells + BC and cells after a 10 min-incubation was compared among non-infected (None), Ad-siNT- and Ad-si01Bcrp-infected day 4 and day 6 SCRH. There was no significant difference in nitrofurantoin accumulation in cells + BC, cells, BEI or *in vitro* $Cl_{biliary}$ between non-infected and Ad-siNT-infected day 4 or day 6 SCRH. Nitrofurantoin accumulation in cells + BC was similar between Ad-si01Bcrp-infected cells and control (Ad-siNT-infected cells), however, cellular accumulation of nitrofurantoin was significantly increased in Ad-si01Bcrp-infected day 4 and day 6 SCRH compared with control (Fig 3. B and C). Nitrofurantoin BEI and *in vitro* $Cl_{biliary}$ values were significantly decreased in Ad-si01Bcrp-infected cells compared to control in both day 4 and day 6 SCRH. In day 4 Ad-si01Bcrp-infected SCRH, BEI and *in vitro* $Cl_{biliary}$ were decreased to ~24% and ~27% of control, respectively, while in day 6 Ad-si01Bcrp-infected SCRH, these values were decreased further to 11% and 14% of control, respectively. The progressive decrease in nitrofurantoin BEI and *in vitro* $Cl_{biliary}$ is in accordance with less Bcrp protein in day 6 than in day 4 Ad-si01Bcrp-infected SCRH (Fig. 2A). These results indicated that knockdown of Bcrp significantly decreased nitrofurantoin BEI and *in vitro* $Cl_{biliary}$ and increased cellular accumulation of nitrofurantoin in SCRH.

Knockdown of Bcrp Did Not Affect Digoxin Biliary Excretion in SCRH

The effect of Bcrp knockdown on the biliary excretion of digoxin, a model P-gp substrate[32], was examined in SCRH. As shown in Fig. 4, knockdown of Bcrp with Ad-si01Bcrp did not affect digoxin accumulation (cells + BC or cells), BEI or *in vitro* $Cl_{biliary}$ in day 4 (Fig. 4 A) or day 6 (Fig. 4 B) SCRH. These results were consistent with expectations that knockdown of Bcrp does not appear to alter the function of other canalicular transport proteins in SCRH.

GF120918 Inhibition of Nitrofurantoin Biliary Excretion in SCRH

Nitrofurantoin accumulation (cells + BC and cells), BEI and *in vitro* $Cl_{biliary}$ in SCRH were compared in the presence and absence of GF120918 in day 4 SCRH. As shown in Fig. 5 (data expressed as % control), GF120918 (2 μM) did not affect nitrofurantoin accumulation in cells + BC (closed bar); however, in the presence of GF120918, cellular accumulation of nitrofurantoin was significantly increased (open bar) similar to accumulation in day 4 Ad-si01Bcrp-infected SCRH. GF120918 (2μM) decreased nitrofurantoin BEI and *in vitro* $Cl_{biliary}$ to ~8% of control values (data not shown).

DISCUSSION

The important contribution of BCRP to the biliary excretion of drugs and metabolites has not been fully appreciated. This may be due to the overlapping substrate specificity of BCRP with other canalicular transport proteins. In the current study, Bcrp was efficiently and specifically knocked down in SCRH by adenoviral vector-mediated RNAi, and the contribution of Bcrp to the biliary excretion of selected drugs was assessed. This is the first *in vitro* model utilizing the intact hepatocyte to specifically assess the contribution of Bcrp to the biliary excretion of drugs.

One prerequisite to successful gene silencing by RNAi is to efficiently introduce siRNA into target cells. Previously, a novel protocol using synthetic siRNA transfected into SCRH was developed in our laboratory to demonstrate the feasibility of applying RNAi in this *in vitro* system; 40 to 50% knockdown of Mrp2 and Mrp3 expression in SCRH was achieved[23]. However rat hepatocytes are primary cells which are, in general, difficult to transfect by traditional methods. Furthermore synthetic siRNA is transient in nature, and it is difficult to achieve complete knockdown of endogenous proteins that have a relatively long half-life, such as canalicular transport proteins. For example, human BCRP protein has a half-life of 35 hours[33], human and mouse P-gp have half-lives of 16 to 50 h, depending on the cell line and culture conditions[34, 35], and rat Mrp2 has a half-life of 27 h[36]. Compared with synthetic siRNA, adenoviral vectors typically yield high infection efficiency even in primary cells. As indicated in Fig. 1A, at MOI of 20, more than 90% infection efficiency was achieved in SCRH. Interestingly, there was a progressive decrease in Bcrp protein levels in Ad-si01Bcrp-infected SCRH (Fig. 2A), which might be due to the relatively long half-life of rat Bcrp in SCRH, similar to that of human BCRP[33]. Such progressive decline in target protein levels also was detected when knocking down radixin in SCRH[37]. Moreover, adenoviral vector-mediated RNAi achieved a long-lasting knockdown effect in SCRH. In day 6 Ad-si01Bcrp-infected SCRH, only trace amounts of Bcrp was detected and such sustained knockdown of Bcrp lasts until at least day 10 (Fig. 2A). Adenoviral vector-mediated RNAi, which could be applied to knock down other canalicular transport proteins, represents a powerful new tool that can be used in SCRH to obtain comprehensive information regarding the contribution of individual transport proteins to drug/metabolite biliary excretion.

Nitrofurantoin, an antibacterial agent widely used in humans to treat urinary tract infections, was transported by BCRP/Bcrp but not by MRP2 or P-gp *in vitro*; in *Abcg2*-knockout mice, nitrofurantoin biliary excretion was almost completely abolished[26]. In the current study, the contribution of Bcrp to nitrofurantoin biliary excretion was assessed in SCRH. Knockdown of Bcrp significantly decreased nitrofurantoin BEI and *in vitro* $Cl_{biliary}$ in day 4 and day 6 SCRH (Fig. 3 B and C). The decrease in nitrofurantoin biliary excretion was due to specific knockdown of Bcrp, since shRNA targeting Bcrp (Ad-si01Bcrp) did not affect the expression of other canalicular transport proteins (Fig. 2 B). These data indicate a predominant contribution of Bcrp to nitrofurantoin biliary excretion in SCRH. The remaining biliary excretion of nitrofurantoin in day 6 Ad-si01Bcrp-infected SCRH may be attributed to residual Bcrp in the cells and/or other canalicular transport protein(s) that play a very minor role in nitrofurantoin biliary excretion in SCRH. These findings are consistent with previously published data[26], and suggest that nitrofurantoin may be used as a probe substrate in SCRH to assess Bcrp function.

The substrate/inhibitor spectrum of BCRP/Bcrp often overlaps with P-gp[38], and the cross-reactivity of chemical inhibitors makes it difficult to identify the contribution of an individual transport protein to the excretion of a test compound, and may confound data interpretation. Digoxin, a typical P-gp substrate, is transported in MDCK II cells over-expressing P-gp, but not MRP2 or Bcrp[39]. However, Wang et al.[40] recently reported that

FTC, a “specific” BCRP inhibitor, also inhibited digoxin transport in Caco-2 cells at a concentration (10 μM) above the IC_{50} previously reported for BCRP(K_i or IC_{50} values of 0.3–1.3μM)[41–43]. Such unexpected results might be explained by the fact that FTC also inhibits P-gp, although to a lesser extent compared to inhibition of BCRP[44]. GF120918 is a widely used P-gp and Bcrp/BCRP inhibitor, however the inhibition of Bcrp function by GF120918 had not been studied in SCRH due to lack of a specific Bcrp probe substrate. In the current study, nitrofurantoin was used as an *in vitro* probe substrate to assess inhibition of Bcrp function by GF120918. In the current study, 2 μM GF120918 almost completely inhibited nitrofurantoin biliary excretion, which is consistent with a pronounced inhibition of Bcrp activity. However GF120918 at this concentration also inhibits P-gp-mediated biliary excretion of digoxin to ~25% of control as previously reported[22]; a similar effect also was observed in the current study (data not shown). Therefore, data obtained utilizing chemical inhibitors needs to be interpreted carefully. Our data clearly indicated that knocking down Bcrp in SCRH did not affect P-gp expression (Fig. 2 B), and in Ad-siBcrp-infected day 4 and day 6 SCRH (Fig 4 A and B), there was no change in digoxin BEI or *in vitro* $Cl_{biliary}$. These results confirm that Bcrp plays a negligible role in digoxin biliary excretion, and that knockdown of Bcrp in SCRH does not interfere with P-gp function. Interestingly, digoxin accumulation in cells + BC and also in cells was lower in day 6 than in day 4 SCRH, suggesting decreased digoxin uptake over time in culture. This finding is consistent with a previous report that Oatp1a4 protein levels in SCRH decreased over days in culture[45] and digoxin is an Oatp1a4 substrate[46]. In contrast, nitrofurantoin accumulation was unchanged in SCRH between day 4 and day 6. The hepatic basolateral transport protein(s) involved in the hepatic uptake of nitrofurantoin have not been identified, although active uptake with saturable, non-saturable and sodium-dependent components have been reported in lactating rats and/or in CIT3 cells[47, 48].

The effect of altered BCRP expression and function on the pharmacokinetics of drugs in clinical studies usually is evaluated by measuring plasma concentrations of drugs[10, 49, 50]; however effects on hepatic drug exposure are almost impossible to measure due to the difficulty of sampling liver tissue in humans. Interestingly, knockdown of Bcrp expression using siRNA and inhibition of Bcrp activity by the chemical inhibitor GF120918 (Fig. 5) both caused a significant increase in cellular accumulation of nitrofurantoin in SCRH. The present *in vitro* data in SCRH imply that hepatocyte concentrations of nitrofurantoin may be increased when Bcrp function is impaired. Nitrofurantoin is hepatotoxic, and increased hepatic concentrations of nitrofurantoin may cause liver injury and elevation of liver enzymes[51–53]. Knockdown of Bcrp in SCRH may serve as a useful *in vitro* model to mimic decreased BCRP function *in vivo* and predict the consequences of impaired Bcrp/BCRP function on the hepatic exposure of drugs/derived metabolites. Further investigations regarding the effects of BCRP knockdown on nitrofurantion disposition in sandwich-cultured human hepatocytes are currently underway.

Application of RNAi in SCRH to knock down Bcrp expression provides a new *in vitro* approach to specifically study the contribution of Bcrp to drug and metabolite disposition in intact hepatocytes. Extrapolating this novel tool to knock down other transport proteins in SCRH and importantly, to apply this approach to sandwich-cultured human hepatocytes, may prove to be an invaluable method to predict the contribution of individual transport proteins to hepatobiliary drug disposition. Recently, our laboratory established the use of sandwich-cultured human hepatocytes to predict the biliary clearance of drugs in humans[54], and demonstrated a good correlation between measured or estimated biliary clearance values in humans and values predicted from *in vitro* data generated in sandwich-cultured human hepatocytes[55].

Transporter-mediated drug-drug interactions (DDIs) in hepatic uptake have been the focus of numerous investigations[56–58], but DDIs in biliary excretion are far less appreciated (package insert of mycophenolate mofetil[59]). Although it is reasonable to hypothesize that drugs excreted primarily by the same canalicular transport protein may exhibit a DDI in biliary excretion, it remains a technical challenge to test this hypothesis. Knock down of canalicular transport proteins such as Bcrp/BCRP via adenoviral vector-mediated RNAi in sandwich-cultured rat hepatocytes in the present study, or in human hepatocytes in the future, represents a novel approach to specifically assess the contribution of a transport protein to the biliary excretion of drugs, and may provide a moderate-throughput approach, suitable for screening purposes, to predict DDIs in biliary excretion and the impact of such interactions on the hepatic exposure of drugs/derived metabolites.

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Nonstandard abbreviations

BC	bile canaliculi
BCRP	breast cancer resistance protein
BEI	biliary excretion index
BSEP	bile salt export pump
CDF	5 (and 6)-carboxy-2',7'-dichlorofluorescein
DDI	drug-drug interaction
GF120918	N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide
FTC	fumitremorgin C

HBSS	Hanks' balanced salt solution
LC/MS/MS	liquid chromatography with detection by tandem mass spectrometry
MDR1	multidrug resistance protein 1
MRP	multidrug resistance-associated protein
P-gp	p-glycoprotein
RNAi	RNA interference
SCRH	sandwich-cultured rat hepatocytes
SCHH	sandwich-cultured human hepatocytes

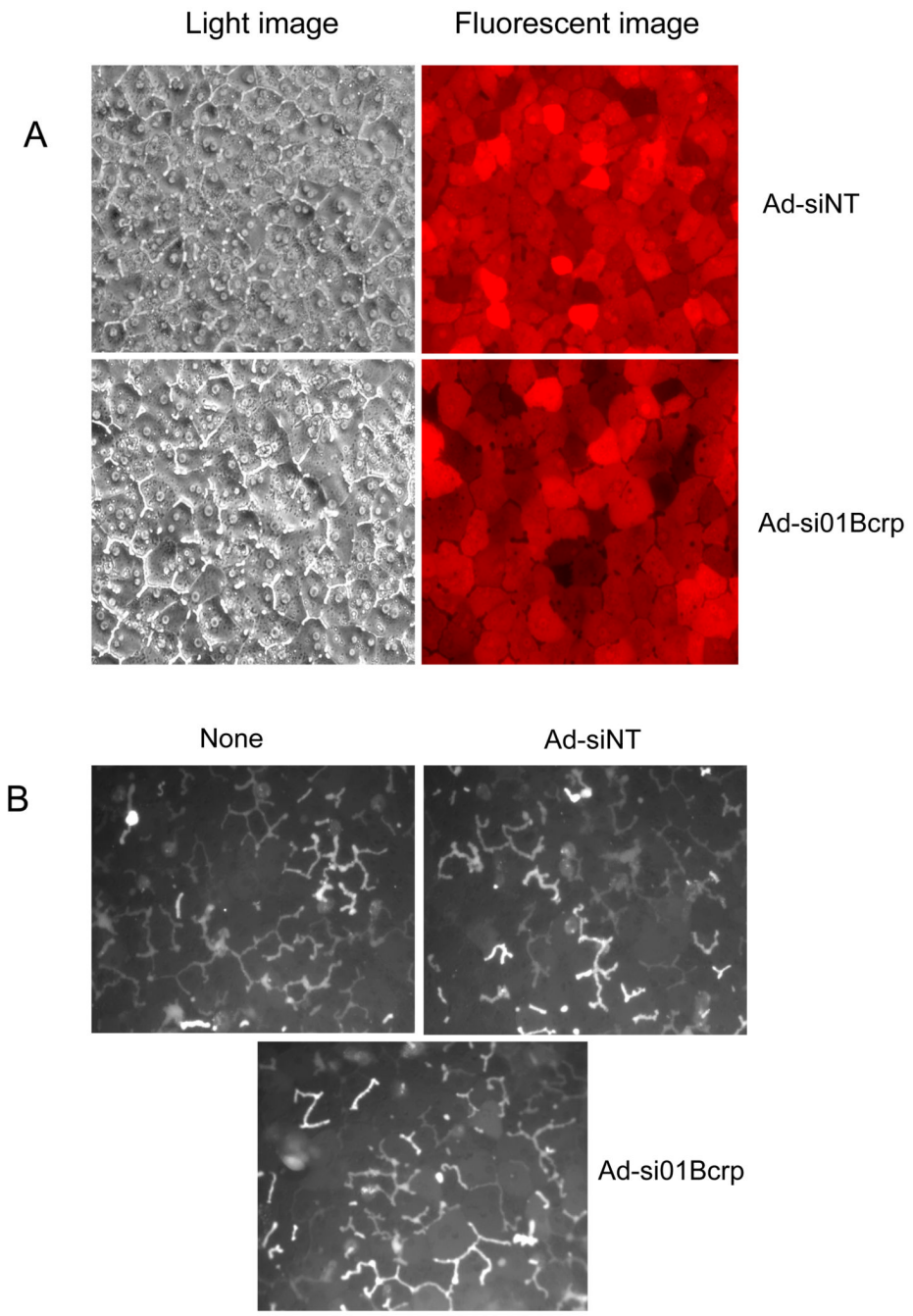

Fig. 1.

Infection of sandwich-cultured rat hepatocytes (SCRH) by adenoviral vectors expressing siRNA. A) High infection efficiency of adenoviral vectors in SCRH. Two hours after seeding, rat hepatocytes were infected with adenoviral vectors (MOI of 20) that expressed non-target control siRNA (Ad-siNT) or siRNA targeting Bcrp (Ad-si01Bcrp). DsRed fluorescent proteins, which were constitutively expressed from these viral vectors after infection, were examined by light microscopy (left panels) and fluorescence microscopy (right panels) to determine infection efficiency in day 4 SCRH. (B) CDF fluorescence in the canalicular networks of day 4 SCRH in non-infected (None), Ad-siNT- and Ad-si01Bcrp-infected hepatocytes after a 10-min incubation with 2 μM CDF diacetate.

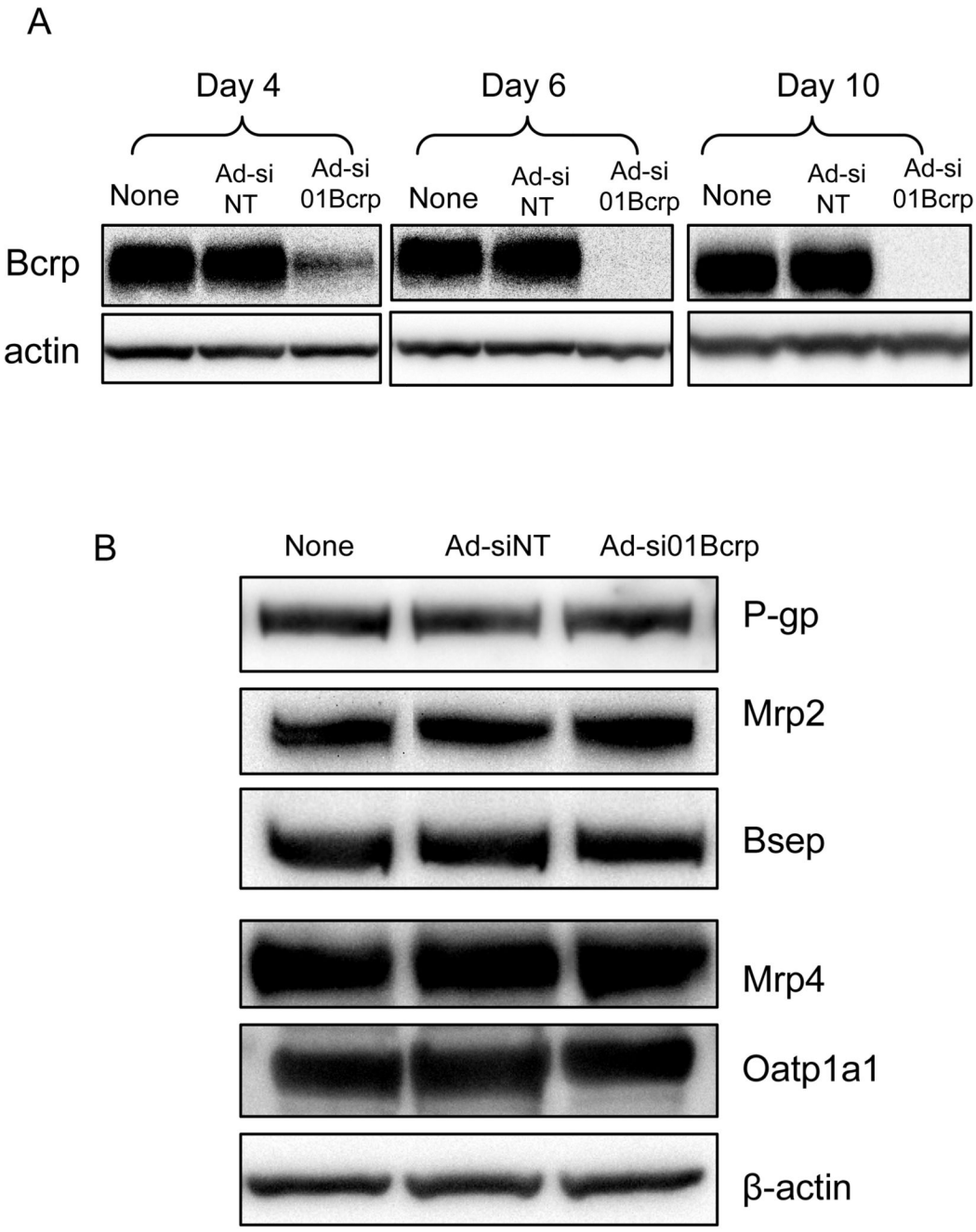

Fig. 2.

Efficient and specific knockdown of Bcrp in SCRH by adenoviral vector-mediated RNAi. A) Bcrp expression in day 4, day 6 and day 10 non-infected (none) SCRH or SCRH infected with Ad-siNT or Ad-si01Bcrp (see Fig.1). Representative results from at least 3 experiments are shown with β-actin as the loading control. B) Cell lysates from day 6 Ad-si01Bcrp-infected SCRH also were blotted for P-gp, Mrp2, Bsep, Mrp4, and Oatp1a1. Representative results from at least 3 experiments are shown.

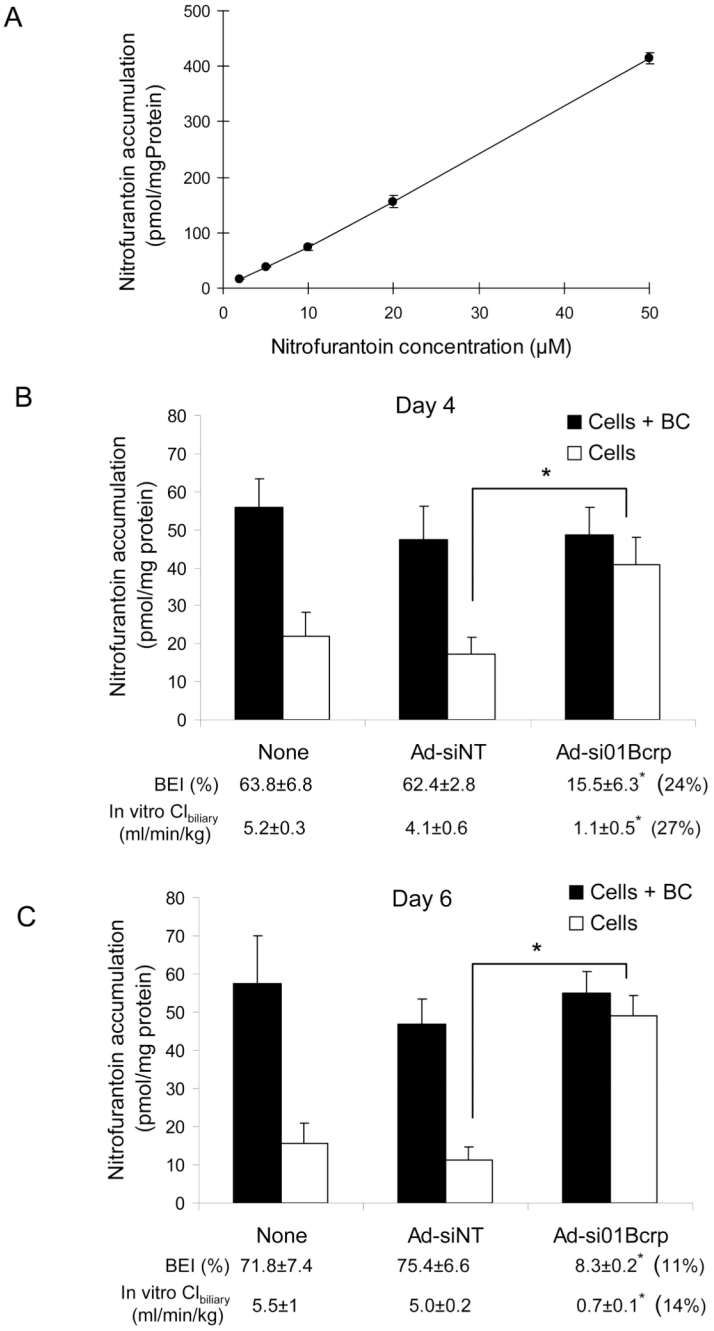

Fig. 3.

Knockdown of Bcrp decreased nitrofurantoin biliary excretion in SCRH. A) Concentration-dependent accumulation of nitrofurantoin in SCRH. Day 4 SCRH were incubated with nitrofurantoin (2–50 μM for 10 min); accumulation [cells + BC (bile canaliculi)] was measured by LC/MS/MS. Data represent mean ± SD of three replicates from one liver. B) and C) Nitrofurantoin accumulation in cells + BC (closed bars) or cells (open bars) after 10-min incubation with 5 μM nitrofurantoin in non-infected (none), Ad-siNT- or Ad-si01Bcrp-infected (see Fig.1) day 4 B), and day 6 C), SCRH. BEI and *in vitro* $Cl_{biliary}$ values are indicated for each group with the percent of control (Ad-siNT) included in parentheses for Ad-si01Bcrp. Data are presented as mean ± S.E.M; n=3 livers in triplicate. * indicates a

statistically significant difference ($p<0.05$) by one-way ANOVA, followed by Dunnett's *t* test compared with control (Ad-siNT).

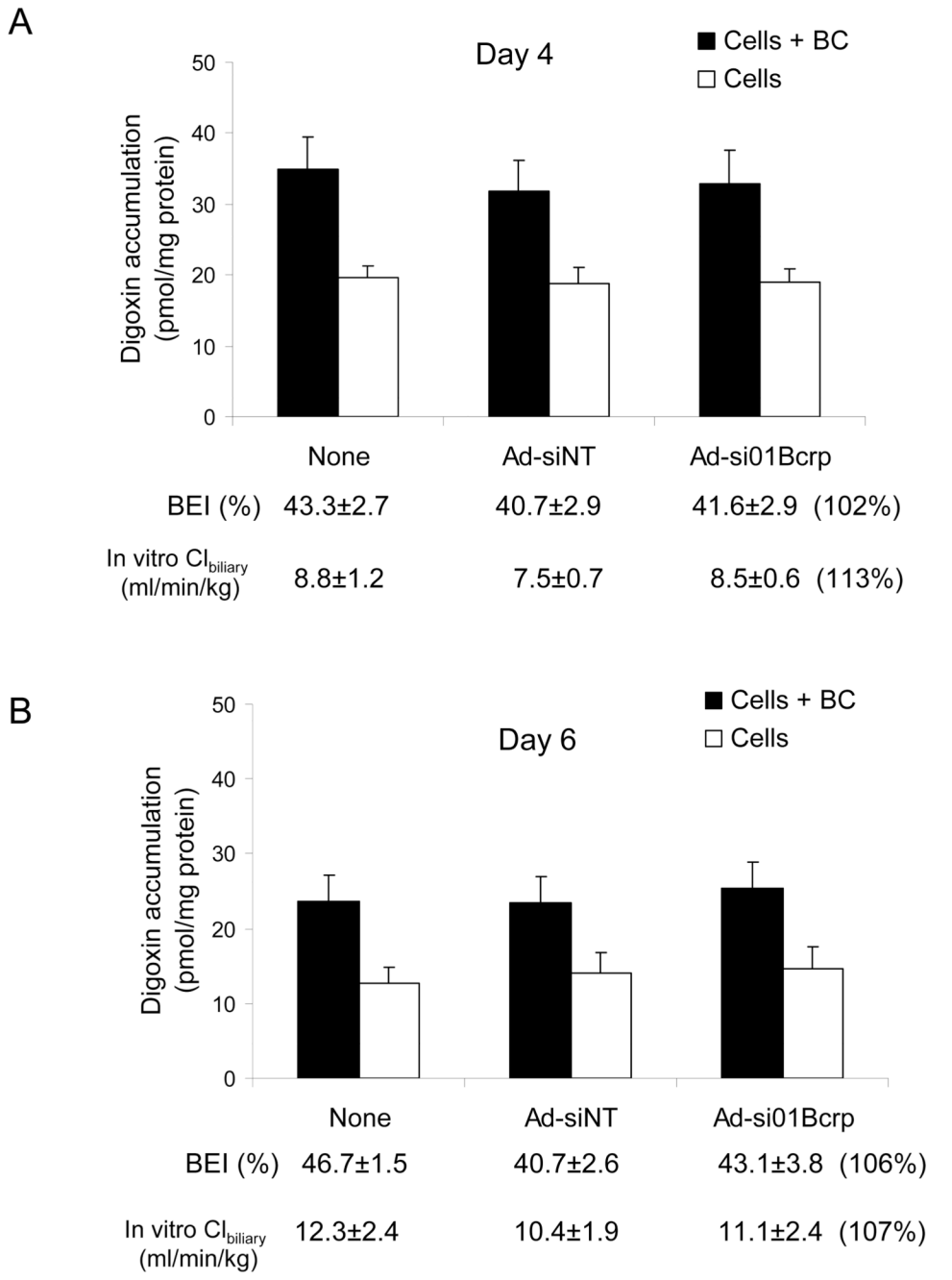

Fig. 4.

Knockdown of Bcrp did not affect digoxin biliary excretion in SCRH. A) and B) Digoxin accumulation in cells + BC (closed bars) or cells (open bars) after 10-min incubation with 1 μM digoxin in non-infected (none), Ad-siNT- or Ad-si01Bcrp-infected (see Fig.1) day 4 A), and day 6 B), SCRH. BEI and *in vitro* $Cl_{biliary}$ values are indicated for each group with the percent of control (Ad-siNT) included in parentheses for Ad-si01Bcrp. Data are presented as mean ± S.E.M; n=3 livers in triplicate.

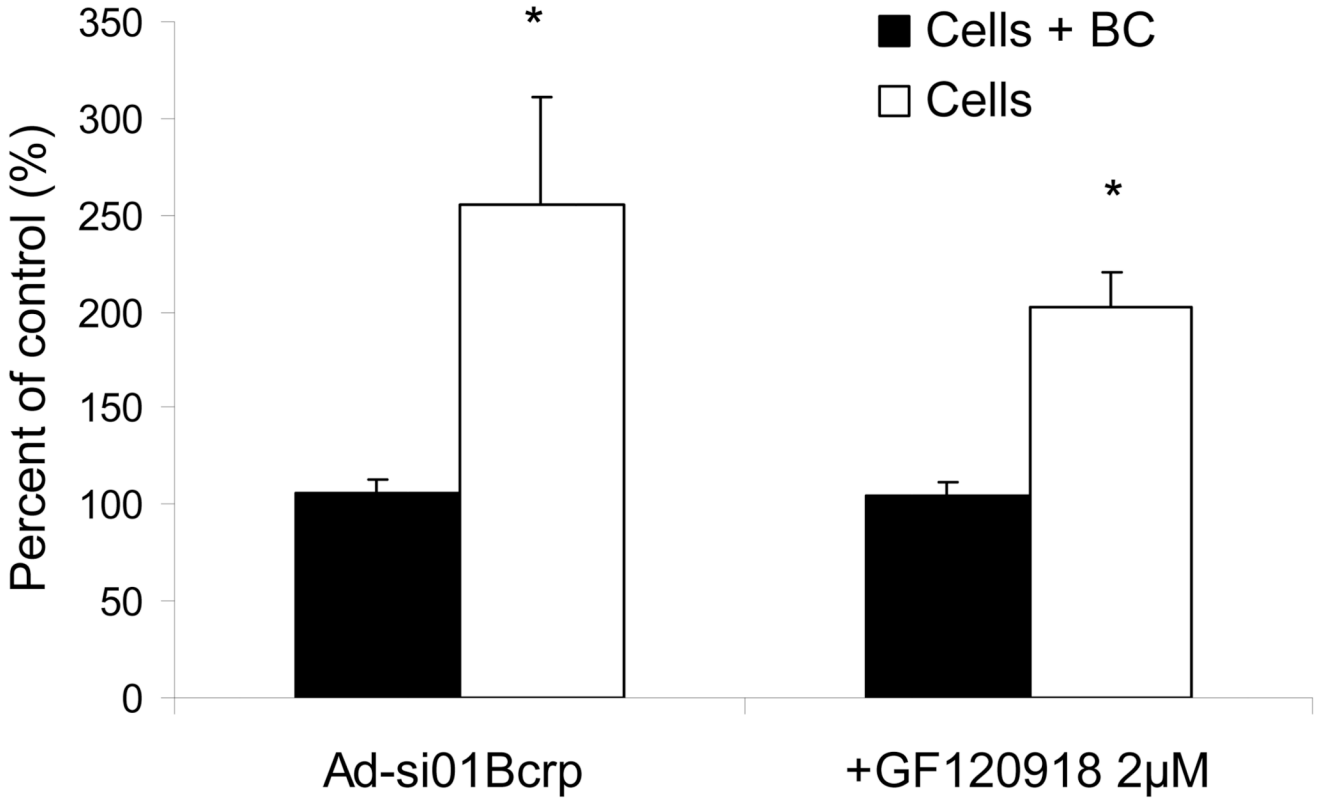

Fig. 5.

Inhibition of nitrofurantoin biliary excretion in SCRH by GF120918. SCRH were incubated with 5 μM nitrofurantoin for 10 min in the presence of 2 μM GF120918 or vehicle control. Nitrofurantoin accumulation in cells+BC (closed bars) or cells (open bars) in the presence of 2μM GF120918 was expressed as percent of control. For comparison, data in Fig 3B for Ad-si01Bcrp are replotted as a percent of control (Ad-siNT). Data are presented as mean ± S.E.M.; n=3 livers in triplicate. * indicates a statistically significant difference ($p<0.05$) by one-way ANOVA.