Generation and Characterization of a Breast Cancer Resistance Protein Humanized Mouse Model


ABSTRACT

Breast cancer resistance protein (BCRP) is expressed in various tissues, such as the gut, liver, kidney and blood brain barrier (BBB), where it mediates the unidirectional transport of substrates to the apical/luminal side of polarized cells. Thereby, BCRP acts as an efflux pump, mediating the elimination of xenobiotics into tissues and it plays important roles in drug disposition, efficacy and safety. Bcrp knockout mice (Bcrp−/−) have been used widely to study the role of this transporter in limiting intestinal absorption and brain penetration of substrate compounds. Here we describe the first generation and characterization of a mouse line humanized for BCRP (hBCRP), in which the mouse coding sequence from the start to stop codon was replaced with the corresponding human genomic region, such that the human transporter is expressed under control of the murine Bcrp promoter. We demonstrate robust human and loss of mouse BCRP/Bcrp mRNA and protein expression in the hBCRP mice and the absence of major compensatory changes in the expression of other genes involved in drug metabolism and disposition. Pharmacokinetic and brain distribution studies with several BCRP probe substrates confirmed the functional activity of the human transporter in these mice. Furthermore, we provide practical examples for the use of hBCRP mice to study drug-drug interactions (DDIs). The hBCRP mouse is a promising model to study the in vivo role of human BCRP in limiting absorption and BBB penetration of substrate compounds and to investigate clinically relevant DDIs involving BCRP.

Introduction

Breast cancer resistance protein (BCRP), also referred to as ABCG2, is a member of the superfamily of ATP-binding-cassette (ABC) transporters, some of which have important roles in the transport of various drugs and their metabolites (Chan et al., 2004; Leslie et al., 2005). BCRP is expressed in many different cell types, such as the luminal membrane of enterocytes, the canicular membrane of hepatocytes, kidney proximal tubule epithelia, the luminal side of the microvascular brain endothelial cells composing the blood-brain barrier (BBB), and the placenta, where it mediates the elimination and can restrict the entry of compounds into tissues (Meyer zu Schwabedissen and Kroemer, 2011). BCRP has attracted attention in drug discovery and development because of its ability to transport xenobiotics into tissues (Meyer zu Schwabedissen and Kroemer, 2011). BCRP has attracted attention in drug discovery and development because of its ability to transport...
many commonly prescribed drugs, such as anticancer agents (e.g., topotecan, doxorubicin, methotrexate, imatinib, sorafenib, or mitoxantrone), the HMG-CoA reductase inhibitor rosuvastatin, the anti-inflammatory drug sulfasalazine, the sympatholytic drug prazosin, and many others (Ni et al., 2010). Furthermore, several BCRP inhibitors, such as ritonavir, omeprazole, imatinib, ivermectin, and curcumin, have been described (Ni et al., 2010; Jani et al., 2011; Kusuhara et al., 2012). Inhibition of BCRP and polymorphic variations associated with altered BCRP activity were shown to result in pharmacokinetic changes of BCRP substrates in the clinic (Zhang et al., 2006; Urquhart et al., 2008; Yamasaki et al., 2008; Hua et al., 2012; Kusuhara et al., 2012). On the basis of its clinical relevance, the International Transporter Consortium (ITC), followed by the Food and Drug Administration (2012) and the European Medicines Agency (2012) regulators, has recommended BCRP as one of the key transporters to be evaluated for substrate and inhibitor interactions during drug development (Giacomini et al., 2010; Zamek-Gliszczynski et al., 2012b).

Such interactions of BCRP with substrates and inhibitors are routinely tested in vitro using overexpression systems or whole cells and, theoretically, physiologically based pharmacokinetic modeling approaches can then help to further predict complex drug-drug interactions (DDIs) (Rostami-Hodjegan, 2012). Despite the great value of these technologies, predicting the effects of transporters on human pharmacokinetics and DDI potential from in vitro data alone remains challenging, and preclinical in vivo studies can provide useful complementary information in this regard. Bcrp knockout mice (Jonker et al., 2002) and, more recently, rats (Zamek-Gliszczynski et al., 2012a) have helped to determine the impact of Bcrp on the pharmacokinetics and tissue distribution of various substrates, including sulfasalazine (Zaher et al., 2006; Zamek-Gliszczynski et al., 2012a), topotecan (de Vries et al., 2007), rosuvastatin (Kitamura et al., 2008), sorafenib (Lagas et al., 2010), daidzein, and genistein (Enokizono et al., 2007).

However, species differences associated with this transporter may limit the use of these knockout models in predicting the role of BCRP in humans (Chu et al., 2013). While most investigations have focused on the differences in the BCRP expression level between animals and man (Lai, 2009; Li et al., 2009; Warren et al., 2009; Uchida et al., 2011; Chu et al., 2013), a few studies have also reported species differences in BCRP substrate specificity or interaction with inhibitors (Li et al., 2008; Gonzalez-Lobato et al., 2010; Mazur et al., 2012). Although the amino-acid sequences between mouse Bcrp and human BCRP are 81% identical and 86% homologous and therefore relatively conserved (Doyle and Ross, 2003), these proteins vary in more than 90 amino acids. As a single amino acid polymorphism in human BCRP can significantly alter its transport efficiency compared with the wild-type (WT) protein (Lee et al., 2007; Urquhart et al., 2008), differences in the kinetics of BCRP-mediated drug transport between human and mouse are very likely. Accordingly, to study the in vivo role of the human instead of the mouse transporter under preclinical conditions, a BCRP-humanized mouse (hBCRP) model would be of great value.

In the present work we describe the generation and extensive characterization of such a humanized mouse model, expressing the human in lieu of the mouse transporter under control of the murine Bcrp promoter. We determined the mRNA and protein expression, evaluated potential compensatory changes in the expression of other genes involved in drug disposition, assessed functional activity of BCRP, and conducted proof-of-concept studies for using the hBCRP mice for DDI assessment.

Materials and Methods

Chemicals and Reagents

Ultra High-Performance Liquid Chromatography–Tandem Mass Spectrometry Protein Quantification. Hanks’ balanced salt solution, HEPES, Tris-HCl, and sodium phosphate (N₂H₂PO₄) were acquired from Sigma-Aldrich (Saint Quentin Fallavier, France). Reagents used for plasma membrane isolation and protein digestion, NaCl, MgCl₂, KCl, sucrose, EDTA, guanidine-HCl, dithiothreitol, iodoacetamide, bovine serum albumin, dextran (molecular weight 70,000), and urea also came from Sigma-Aldrich. Complete Mini (EDTA-free) Protease Inhibitor Cocktail tablets were purchased from Roche (Bâle, Switzerland). Chloroform (HiPerSolv, Chromanorm for HPLC) was supplied by VWR (Strasbourg, France). High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased at Merck (Nogent-sur-Marne, France). Formic acid (99% w/w), HPLC grade, was supplied by Fischer Scientific (Illkirch, France). All the water was prepared with a Milli-Q water purification system (Millipore, Molsheim, France). Sequencing-grade modified trypsin, mass spectrometry–grade rLys-C and ProteaseMAX surfactant were from Promega (Charbonnières-les-Bains, France). The measurement of protein concentration was carried out by using the Micro BCA Protein Assay Kit (Thermo Scientific). Standard solutions of peptides were provided by Pr. M. Vidal and Dr. W. Q. Liu (UMR 8638, Chimie organique médicale et extractive—Toxicologie expérimentale) or by Pepscan (Lelystad, The Netherlands). The accurate concentration of each standard solution was determined, after acid hydrolysis and amino-acid analysis by Dr. E. Thioulouze (Laboratoire de Biochimie, Hôpital Trousseau, Paris, France) or by Pepscan (Lelystad, The Netherlands).

Sulfasalazine/Ko143 Interaction, Daidzein, Genistein, Rosuvastatin, and Topotecan In Vivo Studies. Sulfasalazine, daidzein, genistein, and topotecan were purchased from Sigma-Aldrich (St. Louis, MO) and Ko143 from Enzo Life Sciences (Plymouth Meeting, PA). Rosuvastatin was obtained from TSZ Chem (Framingham, MA). 1-Methyl-2-phenylindole (NMP), solutol (Kolliphor HS 15), thonyrolfluorocacote (TFFA), dimethylsulfoxide, and Tween-20 were obtained from Sigma-Aldrich (St. Louis, MO). Saline was obtained from Baxter Healthcare Corporation (Deerfield, IL). Ammonium acetate and glacial acetic acid were obtained from Fisher Scientific (Waltham, MA).

Positron Emission Tomography Study. Tariquidar dimesylate and Ko143 for the positron emission tomography (PET) study were obtained from Xenova Ltd. (Slough, UK) and Axon Medchem BV (Groningen, The Netherlands), respectively.

Generation of hBCRP and Bcrp Knockout Mice

hBCRP and Bcrp knockout (Bcrp−/−) mice were generated by Taconic Biosciences GmbH (Cologne, Germany) as described below. DNA constructs and cloning: For targeting the Bcrp gene locus, basic vectors containing (1) neomycin and puromycin expression cassettes flanked by frt and f3 sites, respectively, (2) an ~5-kb genomic sequence upstream of the translational start ATG of the mouse Bcrp gene on exon 2 and an ~7-kb genomic sequence downstream of the stop codon on exon 16 used as targeting arms for homologous recombination were constructed. The final targeting vector shown in Fig. 1B was generated by fusing a genomic fragment from start ATG to stop codon of human BCRP in frame to the aforementioned targeting arms by subcloning the fragments via consecutive red/ET recombining (Zhang et al., 1998) into the bacterial artificial chromosome RP11-183N11 (Source BioScience, Nottingham, UK) containing the genomic sequence of human BCRP. The coding exons 2–16 from this clone were sequenced.
and confirmed to match the human BCRP reference sequence (http://
www.uniprot.org/uniprot/Q8UNQ0).

**Generation and Molecular Characterization of Targeted Embryonic Stem Cells.** Culture and targeted mutagenesis of embryonic stem (ES) cells were carried out as described previously (Bhringer et al., 2014). The targeting vector was linearized with NotI and electroporated into a C57BL/6NTac mouse ES cell line. Of 256 G418 and puromycin-resistant ES cell colonies screened by standard Southern blot analyses, 14 correctly targeted clones were identified, four of which were expanded and further analyzed by Southern blot analyses with 5’ and 3’ external probes and internal probes. All clones were confirmed as correctly targeted at both homology arms without additional random integrations (data not shown).

**Generation and Molecular Characterization of hBCRP and Bcrp<sup>−/−</sup> Mice.** For the generation of hBCRP mice, three correctly targeted ES cell clones were expanded, injected into BALBc-blastocysts, and transferred into foster mothers as described previously (Bhringer et al., 2014). Litters from these foster mice were inspected visually and chimerism was determined by hair color. Highly chimeric animals obtained from one of the three correctly targeted clones were used for breeding to an efficient flipase (FLP) deleter strain carrying a transgene that expresses FLP in the germ line in order to delete the neomycin and puromycin expression cassettes in the offspring (Fig. 1D). Offspring from these cross were analyzed by polymerase chain reaction (PCR) to identify heterozygous BCRP-humanized mice. These heterozygous mice were either crossed with each other to generate homozygous hBCRP mice or crossed to a deleter strain expressing a Cre-recombinase in the germ line to delete the human BCRP exons 3–15 (Fig. 1E). Offspring from these crosses were analyzed by PCR to identify homozygous Bcrp knockout mice, which were then further crossed to generate homozygous Bcrp<sup>−/−</sup> mice. The FLP- and Cre-deleter strains mentioned above were generated in house on a C57BL/6NTac genetic background.

**Animal Husbandry and Experimentation**

hBCRP and Bcrp<sup>−/−</sup> mice were bred at Taconic Biosciences GmbH (Cologne, Germany) to obtain homozygous age-matched mice, and age- and sex-matched C57BL/6NTac WT controls were obtained from Taconic Biosciences, Inc. (Hudson, NY). These three mouse lines are maintained and available through Taconic Biosciences. Animals were allowed to acclimatize for at least 5 days prior to an experimental procedure at all experimental locations. Mice were kept in agreement with local laws and regulations and in temperature-controlled environments with 12-hour light/dark cycles and given standard diets and water ad libitum. All animal procedures were approved by local institutional animal care and use committees.

**Quantitative Reverse Transcriptase PCR**

Details on the preparation of mRNA, the synthesis of cDNA, the primers used, and the methods of data analysis for reverse transcription quantitative PCR (RT-qPCR) are provided in the Supplemental Materials and Methods.

**Affymetrix Expression Profiling (Microarray Analysis)**

Details on the preparation of mRNA, quality and quantity determination, and cDNA synthesis used GeneChip Array and data processing methods are provided in the Supplemental Materials and Methods.

**Cortical Microvessel Isolation, Preparation of Plasma Membrane Fraction from Different Tissues, Protein Digestion, and Quantification by Ultra-Performance Liquid Chromatography—Tandem Mass Spectrometry**

Details on the isolation of brain microvessels, preparation of plasma membrane fraction from microvessels, liver, and kidney, protein digestion, and quantification by ultra high-performance liquid chromatography—tandem mass spectrometry (UHPLC—MS/MS) are provided in the Supplemental Materials and Methods.

**Sulfasalazine, Daidzein, Genistein, Rosuvastatin, and Topotecan In Vivo Studies**

Details on the sulfasalazine, daidzein, genistein, rosuvastatin, and topotecan pharmacokinetics studies and the sulfasalazine/Ko143 interaction study and corresponding LC—MS/MS and pharmacokinetic analyses are provided in the Supplemental Materials and Methods.

**PET Study**

Details on general procedures, animal handling, PET imaging, metabolite analysis, and statistical analysis are provided in the Supplemental Materials and Methods.

**Statistical Analysis**

Student’s t test or one-way analysis of variance were applied to determine statistical differences in pharmacokinetics and sulfasalazine/Ko143 interaction in WT, Bcrp<sup>−/−</sup>, and hBCRP mice. Values were considered statistically different when P < 0.05. Analyses were done using Microsoft Excel or GraphPad Prism 5.

**Results**

**Generation of hBCRP and Bcrp<sup>−/−</sup> Mice**

hBCRP mice were generated by a knockin strategy as depicted in Fig. 1, such that the coding sequence of mouse Bcrp from its start codon on exon 2 to its stop codon on exon 16 was replaced by the corresponding genomic human region in mouse ES cells (Fig. 1, A–D). As a result of this approach the human instead of mouse transporter is expressed under control of the mouse Bcrp promoter. Transgenic mice were generated from correctly targeted ES cells. The neomycin and puromycin expression cassettes used for ES cell clone selection were deleted via FLP-recombinase–mediated recombination in vivo by crossing hBCRP transgenic mice with a mouse line expressing Flp-recombinase in the germ line (Fig. 1D). Functional inactivation of the BCRP gene was achieved by crossing hBCRP mice with a mouse line expressing Cre-recombinase in the germ line resulting in a deletion of human BCRP exons 3–15 (Fig. 1E). Homozygous hBCRP and Bcrp<sup>−/−</sup> mice obtained by breeding appeared normal, could not be distinguished from WT animals, and had normal survival rates and fertility.

**Human BCRP and Mouse Bcrp mRNA Expression in WT, hBCRP, and Bcrp<sup>−/−</sup> Mice**

To confirm expression of human BCRP mRNA in different tissues of the hBCRP mice, a TaqMan analysis was conducted on liver, kidney, duodenum, ileum, jejunum, colon, heart, lung, testis, and whole brain samples of WT, hBCRP, and Bcrp<sup>−/−</sup> male and female mice, using a mouse Bcrp- and a human BCRP-specific TaqMan assay (n = 3 mice per sex and genotype). The human BCRP mRNA was detectable in all tissues of the hBCRP mice, but not in WT or Bcrp<sup>−/−</sup> animals. On the basis of cycle threshold values, the highest expression was observed in the kidney, followed by ileum, duodenum and jejunum, colon, testis, liver, brain, lung, and heart (Supplemental Table 1). The same pattern of expression was observed for mouse Bcrp in WT mice, when a murine-specific Bcrp
TaqMan assay was used (Supplemental Table 1). Compared with murine Bcrp mRNA in WT animals, human BCRP in the hBCRP mice was expressed at slightly lower levels, with some variability between organs. While the Bcrp/BCRP expression in the brain was almost identical between WT and hBCRP mice, the human BCRP expression in the kidney and the testis of the hBCRP mice was only ∼30% of the murine Bcrp expression in the WT. All other values varied between these two extremes (Fig. 2). In general the expression level of mouse Bcrp in WT animals was similar between both sexes for most organs, except in the liver, where it was 2.5-fold lower, and in the duodenum and ileum, where it as ∼1.5-fold higher in females compared with males. The hBCRP mice showed the same difference in human BCRP expression between males and females for the liver and the ileum, whereas the level was identical between both sexes in the duodenum (Fig. 2). In summary, a robust expression of human BCRP mRNA in the absence of mouse Bcrp mRNA expression was observed in hBCRP mice. The differences in relative expression levels between organs were the same as that of mouse Bcrp in WT mice with similar variations between males and females, albeit the human transcript was expressed at slightly lower levels than its mouse ortholog.

Bcrp/BCRP Protein Quantification in WT and hBCRP Mice

Bcrp/BCRP protein amounts in WT and hBCRP mice were determined in kidney (n = 3 mice per strain), liver (n = 3 mice per strain), and cortical brain vessels (n = 2 mice for WT and n = 3 mice for hBCRP) by UHPLC–MS/MS analysis using peptides specific to human BCRP, or to mouse Bcrp, and additionally peptides that are common to both mouse and human Bcrp/BCRP (Supplemental Table 2). The plasma membrane marker Na+/K⁺ ATPase was used as a control to evaluate the extraction and digestion homogeneity between each tissue sample, as previously proposed (Hoshi et al., 2013). The homogeneity of liver and kidney samples was confirmed by the low variability of Na⁺/K⁺ ATPase amounts with coefficients of variation (%CV) of 8.9 and 24.9% in WT mice and 8.6 and 13.6% in hBCRP mice, respectively (Table 1). Cortical vessels were less homogenous owing to the low protein amounts available for the assay of each sample (%CV = 31.9% in WT and 32.9% in hBCRP mice). No significant differences in Na⁺/K⁺ ATPase expression levels were observed in any sample between WT and hBCRP mice (Table 1).

The peptide specific to human BCRP was detected in all samples of hBCRP but not WT mice, while the opposite was the case for the mouse Bcrp-specific peptide (data not shown). For direct comparison of protein amounts between WT and hBCRP mice we used the data obtained with the common Bcrp/BCRP peptide (Table 1). Consistent with the mRNA analysis, the highest Bcrp/BCRP protein expression was observed in kidney (WT = 37.7, hBCRP = 9.34 fmol/μg of protein), followed by liver (WT = 1.55, hBCRP = 0.73 fmol/μg of protein), and brain cortical vessels (WT = 0.23, hBCRP = 0.39 fmol/μg of protein). The average Bcrp/BCRP protein amount in hBCRP mice was 4-fold lower in kidney (P < 0.001), 2.1-fold lower in liver (P < 0.001), and 1.7-fold higher in...
brain vessels (statistically not significant) compared with WT controls, which overall is in reasonable agreement with the mRNA measurements.

Assessment of Hepatic Gene Expression Changes in $\text{Bcrp}^+/−$ and hBCRP Mice

Potential compensatory gene expression changes in liver of $\text{Bcrp}^+/−$ and hBCRP mice relative to WT controls were assessed by microarray analysis. The comparison between $\text{Bcrp}^+/−$ and WT mice revealed a total of 22 unique genes (24 total, including genes that are duplicated within the array) altered by greater than 2-fold ($P < 0.05$) (Supplemental Table 3). As expected, the hepatic expression of mouse $\text{Bcrp}$ was most significantly suppressed compared with WT controls (87-fold decrease). Other expression changes in genes coding for proteins (potentially) involved in drug metabolism and disposition were observed for monooxygenase DBH-like ($\text{Moxd}^1$) (17.6-fold increase in $\text{Bcrp}^+/−$ compared with WT mice), solute carrier 3a1 and 41a2 (2.1 and 2.0–fold increase, respectively), and the cytochrome P450 isoform 2b10 (3-fold decrease). In addition, significant changes were noted in some genes not involved in drug metabolism and disposition, such as lipocalin 2, orosomucoid 2 [which can be involved in plasma protein binding of certain drugs (Silamut et al., 1991)], metallothionein 2, and serum amyloid A3 (8.6, 4.9, 4.7, and 4.2-fold increase, respectively). Relative comparison of hBCRP to WT mice detected four genes as significantly changed. In addition to the 78-fold decrease of hepatic $\text{Bcrp}$, an 8.7-fold increase of $\text{Moxd}^1$ expression was the only change in genes coding for proteins involved in drug metabolism and disposition. Additionally, serpine 2 was increased by 2.6-fold and the transmembrane protein 223 was decreased by 2.6-fold (Supplemental Table 3).

Concentration of Sulfasalazine, Daidzein, Genistein, Rosuvastatin, and Topotecan in Blood and Other Tissues of WT, hBCRP, and $\text{BCRP}^+/−$ Mice

The expression of functional BCRP in hBCRP mice was assessed by comparing the concentration-time profiles of various BCRP probe substrates in blood and different tissues of WT, hBCRP, and $\text{BCRP}^+/−$ mice.

**Sulfasalazine.** Following 5-mg/kg i.v. or 20-mg/kg oral (PO) administration of sulfasalazine, blood concentrations were markedly higher in $\text{Bcrp}^+/−$ than in WT and hBCRP mice (Fig. 3, A and B). In WT mice, blood concentrations were below the limit of quantitation after 2 and 1 hour, for the intravenous and oral dose, respectively. Consequently,
pharmacokinetic analyses and comparisons of WT animals with the two other strains were performed with parameters calculated using concentrations up to 1 or 2 hours (Supplemental Table 4A), as well as on the basis of the complete profiles (up to 6 hours; Supplemental Table 4B) from the other strains.

Blood exposure to sulfasalazine was significantly ($P < 0.05$) increased in Bcrp$^{-/-}$ compared with WT mice after intravenous (8.3-fold) and oral (117-fold) administration (Supplemental Table 4A). Concentrations (and profiles) in hBCRP mice were intermediate between those observed in the WT and Bcrp$^{-/-}$ mice (Fig. 3, A and B), with concentrations measurable up to 6 hours. This was reflected by a modest 2.7-fold higher area under the plasma (or blood) concentration-time curve ($\text{AUC}_{0-2}$ in humanized compared with WT mice following intravenous dosing and 4.6-fold higher $\text{AUC}_{0-6}$ after oral administration. Importantly, sulfasalazine exposure in hBCRP mice was nevertheless significantly ($P < 0.05$) lower than in Bcrp$^{-/-}$ animals (Supplemental Table 4, A and B) after dosing through either route. Clearance in Bcrp$^{-/-}$ mice was 8.4-fold lower than in WT controls, whereas in hBCRP mice, it was only decreased by 2.7-fold.

Daidzein. The plasma concentration of daidzein was slightly increased in Bcrp$^{-/-}$ compared with WT mice over a period of 1 hour after administration of a 5-mg/kg i.v. dose, with a significant 1.5-fold higher plasma concentration at the earliest measured time point (5 minutes after administration). In contrast, the concentration-time profiles in hBCRP and WT mice were comparable (Fig. 4, A and B). The mean brain concentrations of daidzein were significantly higher in Bcrp$^{-/-}$ compared with WT mice at 0.5, 1, and 2 hours after administration, but comparable between WT and hBCRP animals (Fig. 4, C–E). Namely, at the 0.5-hour time point the daidzein mean brain concentration was increased by ~4.7-fold in Bcrp$^{-/-}$ compared with WT and hBCRP mice (Fig. 4C). This difference is also reflected by the significantly higher brain-to-plasma concentration ratio in Bcrp$^{-/-}$ mice (1.13) relative to WT (0.20) and hBCRP (0.11) animals. At the 1- and 2-hour time points daidzein was below the limit of quantification in brain tissue of WT and hBCRP mice but still detectable in Bcrp$^{-/-}$ mice (Fig. 4, D and E).

Genistein. The blood concentration of genistein was higher in Bcrp$^{-/-}$ compared with WT mice (Fig. 5, A–C), with statistically significant 2.2- and 2.2-fold increases in $\text{AUC}_{0-24\text{hrs}}$ following 20-mg/kg i.v. and 50-mg/kg PO administration, respectively (Supplemental Table 5). Furthermore, the $\text{AUC}_{0-6\text{hrs}}$ was 2.0-fold higher in Bcrp$^{-/-}$ mice receiving a 20-mg/kg PO dose of genistein, whereas the blood clearance in these mice after 20-mg/kg i.v. administration was 2.4-fold lower relative to the WT controls (Supplemental Table 5). The Bcrp$^{-/-}$ animals also showed a statistically significant 1.5-fold higher $\text{AUC}_{0-24\text{hrs}}$ and $\text{AUC}_{0-6\text{hrs}}$ following 20- and 50-mg/kg PO doses of genistein compared with hBCRP mice (Supplemental Table 5). Brain concentrations were analyzed 15 and 90 minutes after 20-mg/kg i.v. administration of genistein. Bcrp$^{-/-}$ mice showed significantly higher brain concentrations than WT and hBCRP animals at both time points, with 2.6- and 4.4-fold increases at 15 minutes and 8.1- and 9.9-fold increases at 90 minutes compared with WT and hBCRP mice, respectively (Fig. 5, D and E). Furthermore, the brain-to-blood concentration ratio ($K_{\text{brain}}/K_{\text{pl}}$) values in Bcrp$^{-/-}$ mice were 3.3- and 5.1-fold higher at 15 minutes and 19.0- and 7.6-fold higher at 90 minutes than in WT and hBCRP animals (Fig. 5F). Genistein concentrations were also measured in testis, liver, and kidney following 20-mg/kg i.v. administration, but most differences between WT, Bcrp$^{-/-}$, and hBCRP mice were not significant (data not shown).

Rosuvastatin. Bcrp$^{-/-}$ mice showed statistically significant higher blood concentrations of rosuvastatin compared with WT controls following 6.1-mg/kg i.v. and 13.7-mg/kg PO administration (Fig. 6, A and B), as reflected by 4.2- and 3.1-fold $\text{AUC}_{0-24\text{hrs}}$ and 1.7- and 6.7-fold increases in $C_{\text{max}}$ at these doses, respectively (Supplemental Table 6). In contrast, hBCRP mice showed no statistically significant difference in rosuvastatin blood concentrations in WT animals after intravenous administration and only marginal and statistically insignificant 1.6- and 1.7-fold increases in $\text{AUC}_{0-24\text{hrs}}$ and $C_{\text{max}}$ following oral administration (Fig. 6, A and B, Supplemental Table 6). Brain, kidney, and liver concentrations were also measured at 15 minutes and 1 hour after oral administration, but owing to the small number of animals and high variability between individual mice from each group no

![Fig. 3. Sulfasalazine blood concentration-time profiles in WT, hBCRP, and Bcrp$^{-/-}$ mice. Pharmacokinetic profile after 5-mg/kg i.v. (A) or 20-mg PO (B, C) administration of sulfasalazine to male WT (black diamond), hBCRP mice (dark gray triangle), and Bcrp$^{-/-}$ (light gray circle). Sulfasalazine was administered in the absence (A, B) or presence (C) of the BCRP inhibitor Ko143 (20 mg/kg). Sulfasalazine concentration-time profiles in the presence of Ko143 are represented as dotted lines (C). Values shown are mean ± S.D. of n = 4 mice per strain.](image-url)
significant changes were observed between the different lines (data not shown). The percentage recovery of parent compound in feces and urine after intravenous administration and in urine after oral administration of rosuvastatin was highest in WT and lowest in Bcrp<sup>2/2</sup> mice, with hBCRP animals in between (Supplemental Table 7).

**Topotecan.** Following 1-mg/kg i.v. and PO administration, the topotecan blood concentrations in Bcrp<sup>2/2</sup> mice were higher compared with WT controls (Fig. 7, A and B), with concomitant significant 6.1-fold increases in AUC<sub>0-4h</sub> after oral dosing (Supplemental Table 8). Blood concentrations in hBCRP mice were between these two lines (Fig. 7, A and B), showing a significant 1.5-fold lower AUC<sub>0-4h</sub> than Bcrp<sup>2/2</sup> mice after oral administration (Supplemental Table 8). Compared with WT animals the clearance after intravenous administration was 1.6- and 1.4-fold lower in Bcrp<sup>2/2</sup> and hBCRP mice, respectively. Following oral administration the C<sub>max</sub> in Bcrp<sup>2/2</sup> mice was increased by 5.6- and 1.7-fold relative to WT and hBCRP animals, respectively (Supplemental Table 8). While no significant differences were observed between the three lines in topotecan brain exposure 15 minutes after 1-mg/kg PO administration (Fig. 7C), brain concentrations were moderately but statistically significantly increased by 3.1- and 1.7-fold after 60 minutes in Bcrp<sup>2/2</sup> mice compared with WT and hBCRP animals, respectively (Fig. 7D).

As a consequence of the lower topotecan blood concentrations in WT mice, K<sub>b,brain</sub> values were higher relative to Bcrp<sup>2/2</sup> and hBCRP mice at both time points (Fig. 7E).

**BCRP Inhibition Studies Related to Oral Bioavailability of Sulfasalazine and Brain Penetration of Tariquidar**

The possibility of inhibiting intestinal BCRP activity was tested by treating WT, Bcrp<sup>2/2</sup>, and hBCRP mice with a 20-mg/kg PO dose of Ko143 30 minutes prior to the administration of 20-mg/kg PO sulfasalazine. No significant changes in sulfasalazine blood concentrations, AUC<sub>0-last</sub> and C<sub>max</sub> were observed in Ko143 pretreated relative to untreated Bcrp<sup>2/2</sup> mice (Fig. 3C, Supplemental Table 4B). In contrast, pretreatment with Ko143 increased the blood concentrations of sulfasalazine in WT and hBCRP mice (Fig. 3C), detectable up to 6 hours post-dose. This was associated with significant 25.8- and 6.5-fold increases in AUC<sub>0-1</sub> and 45.6- and 5.3-fold increases in AUC<sub>0-last</sub> in WT and hBCRP mice, respectively (Supplemental Table 4A and B). Although C<sub>max</sub> in WT mice under control conditions could only be estimated from three time points, it was significantly increased from 0.25 µM to 5.36 µM with Ko143. Similarly, C<sub>max</sub> in hBCRP mice was 5.2-fold greater in the presence of Ko143 (Supplemental Table 4B).

We also assessed the functional activity of BCRP/BCRP and its inhibition at the BBB of hBCRP and WT mice in vivo using
positron emission tomography and [11C]tariquidar as radiotracer, which is a metabolically stable substrate of murine and human P-glycoprotein (P-gp) as well as murine Bcrp and human BCRP (Kannan et al., 2011; Bankstahl et al., 2013). We have shown before that [11C]tariquidar can be used to visualize Bcrp functional activity at the murine BBB when used at P-gp-saturating tariquidar plasma-concentration levels (Wanek et al., 2012). Mice underwent [11C]tariquidar PET scans after: 1) pretreatment with vehicle only, 2) pretreatment with unlabeled tariquidar at a dose of 12 mg/kg, which inhibits P-gp at the BBB without inhibiting BCRP/Bcrp, and 3) pretreatment with tariquidar (12 mg/kg), and the BCRP/Bcrp inhibitor Ko143 (10 mg/kg) (Allen et al., 2002), as described previously (Wanek et al., 2012) (Fig. 8). We assessed

![Graph](image_url)

**Fig. 5.** Genistein blood concentration-time profiles and brain and blood concentrations in WT, hBCRP, and Bcrp<sup>−/−</sup> mice. Pharmacokinetic profiles in WT (black circles), hBCRP (black triangles), and Bcrp<sup>−/−</sup> male mice (white circles) following (A) 20-mg/kg i.v., (B) 20-mg/kg PO or, (C) 50-mg/kg PO administration of genistein, respectively. (D and E) Genistein blood (black bars) and brain (gray bars) concentrations (D) 15 minutes and (E) 90 minutes after 20-mg/kg i.v. administration. (F) Genistein brain-to-blood concentration ratio in WT (black bars), hBCRP (gray bars), and Bcrp<sup>−/−</sup> (white bars) mice 15 and 90 minutes after 20-mg/kg i.v. administration. Values shown are mean ± S.D. with n = 8 (A–C) or n = 4 (D–F) mice per strain and time point. *P < 0.05, statistically significant compared with Bcrp<sup>−/−</sup> mice.

![Graph](image_url)

**Fig. 6.** Rosuvastatin blood concentration-time profiles in WT, hBCRP, and Bcrp<sup>−/−</sup> mice. Pharmacokinetic profiles in WT (black diamonds), hBCRP (black triangles), and Bcrp<sup>−/−</sup> male mice (white squares) following (A) 6.1-mg/kg i.v. and (B) 13.7-mg/kg PO administration of rosuvastatin, respectively. Values shown are mean ± S.D. with n = 4 mice per strain and time point.
radiolabeled metabolites of $[^{11}C]$tariquidar in plasma and brain samples collected at the end of PET scanning by radio-TLC analysis. At 60 minutes after $[^{11}C]$tariquidar injection 80\% ($n = 12$) and 85\% ($n = 11$) of radioactivity in plasma and 93\% ($n = 6$) and 97\% ($n = 3$) in brain was in the form of unchanged $[^{11}C]$tariquidar for hBCRP and WT mice, respectively. This suggested that there were no differences in radiotracer metabolism between hBCRP and WT mice. Brain uptake of $[^{11}C]$tariquidar was expressed as $K_b$ of radioactivity in the last PET frame (i.e., at 50–60 minutes after radiotracer injection). In vehicle scans, $K_b$ values were low and not significantly different between hBCRP and WT mice ($K_b$: 1.58 ± 0.38 for WT and 1.50 ± 0.25 for hBCRP). In scans after P-gp inhibition with tariquidar (12 mg/kg), $K_b$ values were only moderately and not significantly increased compared with vehicle-treated animals by 3.6-fold in hBCRP mice ($K_b$: 3.20 ± 0.21, 2.0-fold increase) and WT mice ($K_b$: 3.35 ± 0.31, 2.2-fold increase), which was consistent with functional compensation of P-gp inhibition by BCRP/Bcrp for dual P-gp/BCRP substrates (Kodaira et al., 2010). In scans after P-gp and BCRP/Bcrp inhibition with tariquidar (12 mg/kg) and Ko143 (10 mg/kg), respectively, $K_b$ values were significantly ($P < 0.001$, one-way analysis of variance followed by Bonferroni’s multiple comparison test) increased compared with tariquidar-only treated animals by 3.6-fold in hBCRP mice (Ko143: 11.66 ± 2.16) and by 3.3-fold in WT mice (Ko143: 10.90 ± 0.93). This strongly suggested that hBCRP and WT mice had comparable functional activity of BCRP/Bcrp at the BBB and that this transporter was effectively inhibited by Ko143.

**Discussion**

Here, we describe the generation of a BCRP-humanized mouse model via a sophisticated replacement of the murine $Bcrp$ coding sequence with corresponding human genomic DNA, such that the human transporter is expressed under control of the mouse $Bcrp$ promoter (Fig. 1). The hBCRP mice were healthy and showed no obvious phenotypic abnormalities. Human $BCRP$ mRNA was detected in the expected organs, such as liver, gut, brain, kidney, and testis of humanized mice, albeit at slightly lower levels than mouse $Bcrp$ mRNA in WT mice in most tissues (Fig. 2). The mRNA measurements were further confirmed by protein quantification in selected organs, showing the BCRP amounts to be 2- and 4-fold lower in liver.
and kidney, respectively, and 1.7-fold higher (not statistically significant) in cortical vessels of hBCRP compared with WT mice (Table 1).

Although the hepatic and renal protein amounts of Bcrp and Na⁺/K⁺ ATPase in membrane fractions are within the same magnitude as previously reported by Kamiie et al. (2008) using similar extraction methods on tissues from a ddy WT mouse strain, we observed lower Bcrp levels in the C57BL/6NTac mice used in our study (1.55 versus 8.84 fmol/mg of protein). The most likely explanation for this is an interstrain
difference. When comparing our results for cortical vessels with those previously described in C57BL/6 mice by Sadiq et al. (2015), we measured significantly lower levels in this tissue (0.225 fmol/μg versus 8.69 fmol/μg of protein). However, the method of vessel isolation differed between the two studies, such that Sadiq et al. (2015) obtained capillaries from whole brain lysate and carried out three successive filtrations (210-, 85-, and 20-μm nylon mesh) to enrich in microcapillaries, whereas we used only one 10-μm nylon mesh to filter cortical vessels. Therefore, the lower values can be explained by a dilution of Bcrp/BCRP in our samples. Taking into account that the average BCRP/BCRP protein amount in the human BBB was recently shown to be 1.85-fold greater than in WT mice (Uchida et al., 2011) and that we observed a 1.75-fold higher expression level of human BCRP in cortical vessels of hBCRP mice than murine Bcrp in WT controls (Table 1), we conclude that the expression level of this transporter in hBCRP mice and humans is similar. The comparison of BCRP protein levels between humans and hBCRP mice in other organs requires further investigation.

No compensatory changes in the expression of genes coding for proteins involved in drug metabolism and disposition were observed in liver of hBCRP mice compared with WT controls with the exception of an 8.7-fold increase of Moxd1 mRNA (Supplemental Table 3). The presumably very minor role of Moxd1 in drug metabolism and disposition (Chambers et al., 1998) and the moderate (2.6-fold) change of only two other genes, serpine 2 and transmembrane protein 223, in hBCRP mice are not expected to limit their use to study BCRP-mediated absorption or disposition. Of the expression changes in 22 genes in Bcrp<sup>−/−</sup> mice only an ~2-fold increase in solute carriers 3a1 and 41a2, which don’t appear to play relevant roles in drug transport and 3-fold decrease in the cytochrome P450 isoform 2b10 expression are of potential interest in the context of drug metabolism and disposition (Supplemental Table 3), but these changes do not preclude the general application of these mice. Further investigations will be needed to assess changes in other tissues.

Our studies confirmed the important role of Bcrp/BCRP in oral bioavailability and/or tissue distribution of sulfasalazine, daidzein, genistein, rosuvastatin, and topotecan, as demonstrated by the various differences in pharmacokinetics and brain penetration of these compounds between WT, hBCRP and Bcrp<sup>−/−</sup> mice. Moreover, the functional activity of human BCRP in hBCRP mice was clearly shown by comparing the blood and tissue exposure of these BCRP probe substrates between the three mouse lines. In the case of sulfasalazine, the AUC after intravenous and oral administration was increased significantly more in Bcrp<sup>−/−</sup> than in hBCRP mice compared with WT controls (Supplemental Table 4A). In addition, the clearance and C<sub>max</sub> of sulfasalazine were altered to a much lesser extent in hBCRP than in Bcrp<sup>−/−</sup> mice. Similar observations were made for the plasma or blood concentrations of daidzein (Fig. 4, A and B), genistein (Fig. 5, A–C), and rosuvastatin (Fig. 6, A and B, Supplemental Table 6), which were slightly increased in Bcrp<sup>−/−</sup> but not hBCRP mice relative to WT controls. Furthermore, the mean brain concentration and brain-to-plasma ratio of daidzein and genistein were significantly higher in Bcrp<sup>−/−</sup> compared with WT and hBCRP animals (Fig. 4, C–E, Fig. 5, D–F). Blood concentrations of topotecan in hBCRP mice after intravenous and oral administration as well as brain concentrations 60 minutes after oral dosing were also lower compared with Bcrp<sup>−/−</sup> animals, but the recovery toward WT levels was relatively weak (Fig. 7, A, B, and D, Supplemental Table 8). On the basis of our results the overall relevance of Bcrp/BCRP in restricting the brain penetration of the dual P-gp/BCRP substrate topotecan appears to be relatively low, which is in agreement with a previous study (de Vries et al., 2007) and can be attributed to the activity of P-gp compensating for the loss of Bcrp. Accordingly, the higher K<sub>b,brain</sub> values at 15 and 60 minutes after oral administration in WT compared with Bcrp<sup>−/−</sup> and hBCRP mice (Fig. 7E) are a consequence of the lower topotecan blood concentrations in WT mice, i.e., the relatively stronger effect of Bcrp in limiting oral bioavailability versus brain penetration. In summary, our data demonstrate that human BCRP is able to functionally compensate for the loss of mouse Bcrp in the hBCRP mice.

A consistent trend observed for all five probe substrates is that the blood concentrations in hBCRP mice never reach the levels observed in WT controls. This observation might be generally explained by the lower expression level of the transporter in the gut, liver, and kidney of the humanized mice (Fig. 2), though species differences in the transport activity between murine Bcrp and human BCRP might contribute to this result for some of the compounds. The latter point might also explain why the pharmacokinetic profiles of sulfasalazine, rosuvastatin, and topotecan in hBCRP mice vary in different ways from those observed in WT and Bcrp<sup>−/−</sup> mice. Furthermore, we cannot exclude that changes in the expression level of other transporters or drug metabolizing enzymes contribute to the differences in the pharmacokinetic profiles in the hBCRP mice. However, on the basis of the results from our microarray studies (Supplemental Table 3), such changes appear to be minimal and they are unlikely to be of major relevance. In contrast to the results observed for blood concentrations, daidzein and genistein brain concentrations were fully recovered (Fig. 4, C–E; Fig. 5, D–F), consistent with the similar expression level of Bcrp/BCRP in cortical vessels of WT and hBCRP mice (Fig. 2, Table 1). Interestingly, the topotecan brain concentration in hBCRP mice was 1.8-fold higher (P < 0.05) than in WT mice 60 minutes after oral administration (Fig. 7D). This observation might be attributed to a species difference in the topotecan transport activity of mouse Bcrp versus human BCRP, which could also explain the relatively weak recovery of blood concentrations in hBCRP mice (Fig. 7, A and B).

In addition to assessing substrate interactions with BCRP, studying DDIs caused by the inhibition of this transporter is a potentially valuable application of the hBCRP mouse. To evaluate their utility for this type of application, we investigated the effect of the BCRP inhibitor Ko143 on oral bioavailability of sulfasalazine and brain penetration of tariquidar. Ko143 increased the blood concentrations, AUC<sub>0–last</sub> (and AUC<sub>0–t</sub>), and C<sub>max</sub> of sulfasalazine in WT and hBCRP but not Bcrp<sup>−/−</sup> animals (Fig. 3C, Supplemental Table 4, A and B), whereby the stronger inhibitory effect in WT compared with hBCRP mice might be attributed to the differences in the expression level of Bcrp/BCRP in the gut of these the two mouse lines. In agreement with the comparable expression level of Bcrp/BCRP at the BBB in WT and hBCRP mice, Ko143 increased the K<sub>b,brain</sub> Value of tariquidar to a similar extent in both lines (Fig. 8E).
The present work describes the first generation and extensive characterization of a humanized BCRP mouse model. The results from these studies demonstrate the integrity and functionality of this novel model. The hBCRP mouse provides a valuable alternative or adjunct to WT animals in studies using Bcrp knockout mice or chemical inhibitors of BCRP/Bcrp aiming to assess substrate or inhibitor in vivo interactions with BCRP, specifically where species differences are of concern. Though the results obtained with topotecan might indicate a difference in the topotecan transport activity of mouse Bcrp versus human BCRP (see above), the systematic analysis of species differences by using the hBCRP mice is beyond the scope of this work and will be subject to further investigations. On the basis of previously published work, fumitremorgin C could be considered as a potential inhibitor (Gonzalez-Lobato et al., 2010) and phloretin A or bisphenol A as substrates (Li et al., 2008; Mazur et al., 2012) for such studies. Thus, the hBCRP mouse model provides a novel tool for identifying differences in inhibitor or substrate interactions with this transporter, either for basic research purposes or in drug development. With regard to the latter, this information, in conjunction with results obtained from in vitro studies and in Bcrp−/− mice, can help to estimate the potential need for clinical DDI studies.

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References


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